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USE OF MONOCLONAL ANTIBODIES TO STUDY THE STRUCTURAL BASIS OF THE FUNCTION OF NICOTINIC ACETYLCHOLINE RECEPTORS ON ELECTRIC ORGAN AND MUSCLE AND TO DETERMINE THE STRUCTURE OF NICOTINIC ACETYLCHOLINE RECEPTORS ON NEURONS

Annual Report

Jon M. Lindstrom

March 16, 1989

Supported by

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The Salk Institute Receptor Biology Laboratory
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During the past year we continued studies of nicotinic acetylcholine receptors of both muscle and neuronal types. Muscle-type acetylcholine receptors were studied in a human cell line, in Torpedo electric organ, using cDNAs for subunits of electric organ receptor expressed in yeast, and by chemical synthesis of parts of Torpedo receptor subunits. Neuronal nicotinic receptors extracted from brain membranes were characterized biochemically, fragments of receptor subunits were expressed in bacteria, and in collaborative experiments neuronal nicotinic receptors were localized histologically using monoclonal antibodies. Neuronal alpha-bungarotoxin-binding protein has been used to begin a library of monoclonal antibodies, and two candidates for subunit cDNAs have been sequenced and are being tested.

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ABSTRACT

The purpose of this work is to characterize at molecular and functional levels nicotinic acetylcholine receptors of the types found in muscles and neurons. The particular military significance of these receptors is that many chemical warfare agents act indirectly or directly through these receptors. These agents can disrupt cognitive behavior and kill by paralyzing respiratory muscles.

Substantial progress was made during the past year on studies of the distinct, but genetically related, nicotinic receptors characteristic of muscle and neurons.

The human cell line TE671, which we had previously found to synthesize muscle-type receptors, was found to have the unusual property of synthesizing an amount of a synthetic intermediate of α subunits nearly equal to the amount of native receptors that they synthesize. This synthetic intermediate has a nearly native conformation of its main immunogenic region, but has very low affinity for small cholinergic ligands.

Subunits of the muscle-type nicotinic receptors from Torpedo electric organ were expressed from their cloned cDNAs in transformed yeast. The α subunits assumed a conformation similar to that of a synthetic intermediate identified in muscle cells, but did not properly assemble with other subunits to form native receptors.

Specific peptide fragments of the α subunits of receptors from Torpedo electric organ and human muscle were chemically synthesized and found to bind weakly, but specifically, to some monoclonal antibodies directed at the main immunogenic region, thereby localizing some of the amino acids that form this structure.

Neuronal nicotinic receptors extracted from the brains of chickens, rats, and cattle were shown to be composed of equal numbers of acetylcholine-binding subunits and structural subunits. This proves that neuronal nicotinic receptors cannot have the pentagonal symmetry of muscle-type nicotinic receptors, which have the subunit composition $\alpha_2\beta\gamma\delta$. It seems most likely that neuronal nicotinic receptors have the subunit composition A_2S_2.

Developmental expression of neuronal nicotinic receptor subtypes in chickens was studied using immunochemical and molecular genetic techniques. Unique fragments of several neuronal nicotinic receptor subunit cDNAs have been expressed as proteins in bacteria in high yield, purified, and used as antigens to make specific antisera.

Histological studies of neuronal nicotinic receptors using monoclonal antibodies prepared by us were conducted by our collaborators studying frogs, finches, and chickens. These studies localized receptors, in some cases in pre- and extrasynaptic locations.

Neuronal α-bungarotoxin-binding proteins from chicken and rat brains were used as immunogens to begin a library of monoclonal antibodies. These monoclonal antibodies will be used to characterize the structure of these proteins, as we have characterized the structure of neuronal nicotinic receptors. Two candidate cDNAs for α-bungarotoxin-binding protein subunits have been cloned and sequenced, and are being tested for their identity.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

For the protection of human subjects, the investigators have adhered to policies of applicable Federal Law 45CFR46.

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INTRODUCTION

Significance of Nicotinic Receptors

Most "nerve gas" chemical warfare agents inhibit acetylcholinesterase. This causes acetylcholine released at nerve endings to persist for long periods at high concentrations, which first excessively activates acetylcholine receptors and then inactivates them by desensitization. Some of these agents may also directly impair receptor function. Death can result from paralysis of respiratory muscles. Effects on both acute and chronic brain function may be severe, but are less well characterized.

Medically, short-acting nicotinic receptor antagonists are used as muscle relaxants during surgery. Autoantibodies to nicotinic receptors cause the weakness characteristic of myasthenia gravis. Neuronal nicotinic receptor amounts are also reduced in Alzheimer's and Parkinson's diseases, although this is probably a secondary manifestation of the disease. Nicotine acts on neuronal nicotinic receptors to produce the pleasure experienced by and addiction characteristic of tobacco smokers. Some insecticides (of which nicotine is an example) can act directly on nicotinic receptors, and other insecticides can act like nerve gases to paralyze respiratory muscles. Venoms from cobras, kraits, frog skins, and some corals contain toxins that block nicotinic receptor function.

Muscle-type nicotinic receptors[1] are the best-characterized neurotransmitter receptors and also the best-characterized membrane ion channels. In addition to their intrinsic significance, they serve as a model for the study of other receptors and channels.

Neuronal nicotinic receptors[2] are only beginning to be studied at the molecular level through the use of newly available monoclonal antibody (mAb) and complementary DNA (cDNA) probes. The roles of neuronal nicotinic receptors in normal, much less pathological, brain function is not well understood.

Nicotinic acetylcholine receptors of muscles and nerves are part of a gene family which also includes receptors for glycine[3] and gamma-aminobutyric acid.[4] There are probably other members of this ligand-gated ion channel family. This family is distinct from the gene family of G-protein-associated receptors, which includes muscarinic receptors, adrenergic receptors, and rhodopsin.[5,6] The ligand-gated ion channel family is also distinct from the voltage-gated ion channel family, but there are interesting structural similarities between the ion channels formed by these genetically unrelated proteins.[7]

Overview of our Studies During the Past Three Years

My laboratory has been studying nicotinic acetylcholine receptors for more than 15 years. Initially, receptors from fish electric organs were studied, and later, also receptors from mammalian skeletal muscle. Antibodies, and especially mAbs, proved to be extremely useful probes. By the start of this contract, we had found that some of the mAbs we had prepared to receptors from fish electric organs could be used to identify and, for the first time, purify neuronal nicotinic receptors.[8] In 1987, we reviewed in some detail studies of neuronal nicotinic receptors to that date,[2] and we more briefly reviewed studies of muscle nicotinic receptors. More recently we have reviewed these areas, and especially our studies, in several brief articles.[9-11]

Figure 1 compares the subunit structures of nicotinic receptors of the muscle and neuronal types, and summarizes some aspects of our molecular characterization of these receptors. Receptors from the electric organs of Electrophorus and Torpedo and from vertebrate skeletal muscle are composed of four kinds of subunits termed α, β, γ, and δ, in order of increasing apparent molecular weight. Acetylcholine (ACh) binding sites are formed by the α subunits, as shown by affinity labeling experiments with 4-(N-maleimido)benzyltrimethylammonium iodide (MBTA) and other reagents.[12] These receptors have high affinity for α-bungarotoxin (αBgt) and micromolar affinity for nicotine. They are composed of two α subunits and one of each of the other three kinds of subunits. The subunits are organized like barrel staves in the order $\alpha\beta\alpha\gamma\delta$ around a central ion channel.[13,14] The cDNA sequences of the α, β, γ, and δ subunits reveal substantial homology, indicating that all of these subunits evolved from a common precursor and that all of the subunits probably have a basically similar shape and transmembrane orientation of their polypeptide chains.[15,16]

	EEL ELECTRIC ORGAN and MUSCLE	CHICK BRAIN		RAT BRAIN	HUMAN and BOVINE BRAIN
ACh binding subunits		— 75kD*	cDNA "α4"	— 79kD*	— 75kD*
structural subunits	δ — 59kD	— 59kD*			
structural subunits	γ — 52kD	—	— 49kD	cDNA "β2" — 51kD	— 50kD
ACh binding subunit	β — 48kD α — 40kD*				
αBgt binding	+	−	−	−	−
nM Nicotine binding	−	+	+	+	+
MBTA labeling	*	*	*	*	*
subunit stoichiometry	$\alpha_2\beta\gamma\delta$	x_2y_2		x_2y_2	x_2y_2

Figure 1. Summary of immunoaffinity purification and characterization of neuronal nicotinic receptors. Adapted from reference 2.

We found that the human cell line TE671 produces large numbers of functional muscle-type nicotinic receptors.[17-19] These receptors were purified and shown to consist of α, β, γ, and δ subunits.[19] cDNAs for their α and δ subunits were cloned and sequenced,[18,19] and we are in the process of sequencing cDNA clones for the β and γ subunits. For the first time, this provided a uniform source of relatively large numbers of human muscle nicotinic receptors for electrophysiological, pharmacological, biochemical, and molecular genetic studies.

Using a mAb to receptors from Electrophorus electric organ, we immunoaffinity purified nicotinic receptors from chicken brains.[8,20] Unlike receptors from muscle, these did not bind α-bungarotoxin and had very high affinity for nicotine.[21] Also, unlike receptors from muscle, these receptors were found to consist of only two kinds of subunits, structural subunits of apparent molecular weight 49,000 and acetylcholine-binding subunits of 59,000 apparent molecular weight. Using mAbs to these receptors, a second subtype was found to exist in chicken brains in nearly equal amounts.[20] This subtype used apparently identical structural subunits, but had acetylcholine-binding subunits of 75,000 apparent molecular weight.

Using a mAb to receptors purified from chicken brains, a single subtype of receptor accounting for >90% of the high-affinity nicotine binding sites in extracts was purified from rat brains.[22] It was composed of structural subunits with an apparent molecular weight of 51,000 and acetylcholine-binding subunits with an apparent molecular weight of 79,000.

Using mAbs to receptors purified from rat brains, similar receptors were identified in human brains and purified from bovine brains.[23]

Determination of N-terminal amino acid sequences of subunits from chicken and rat brains permitted identification of corresponding subunit cDNAs (Figure 2).[24-26] We cloned and sequenced cDNAs for the chicken brain structural subunit[25] and the 75,000 apparent molecular weight acetylcholine-binding subunit.[26] These sequences revealed homologies to other members of the nicotinic receptor gene family, especially to subunits of similar receptors from rat brain (Figure 3).[27-32] Like all members of this gene family, these subunits exhibited such features as four conserved, hydrophobic, putative transmembrane domains, and a poorly conserved large putative cytoplasmic domain. The acetylcholine-binding subunits exhibited a cysteine pair at positions 192,193, corresponding to the sites at which the affinity labeling reagent MBTA is known to react with receptors from Torpedo.[12]

Several groups have now identified in rats[27-30,33] and chickens[25,26,32] a family consisting of several neuronal nicotinic receptor subunit cDNAs for both the acetylcholine-binding and structural subunits. The muscle nicotinic receptor α

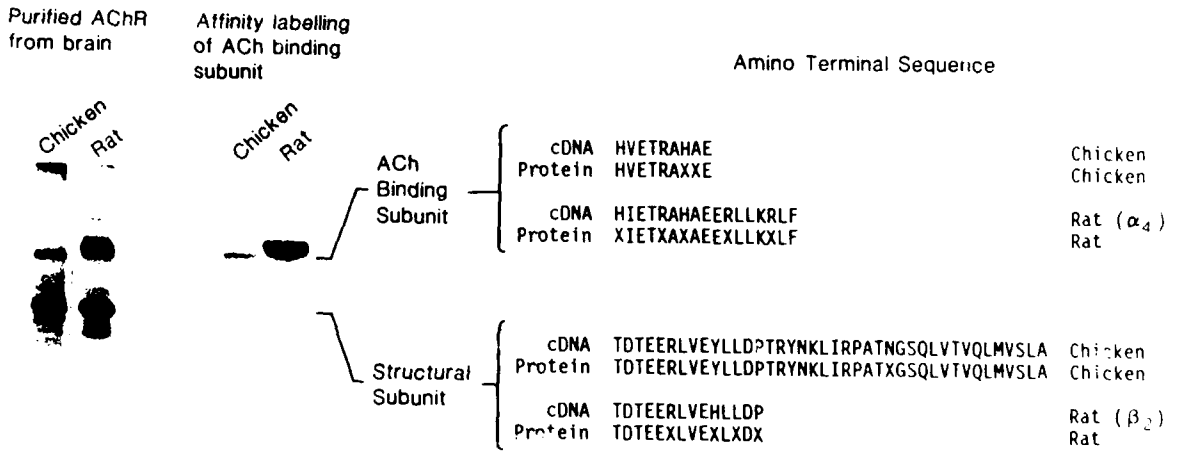


Figure 2. Structure and N-terminal amino acid sequences of brain nicotinic receptors. Reproduced from reference 11.

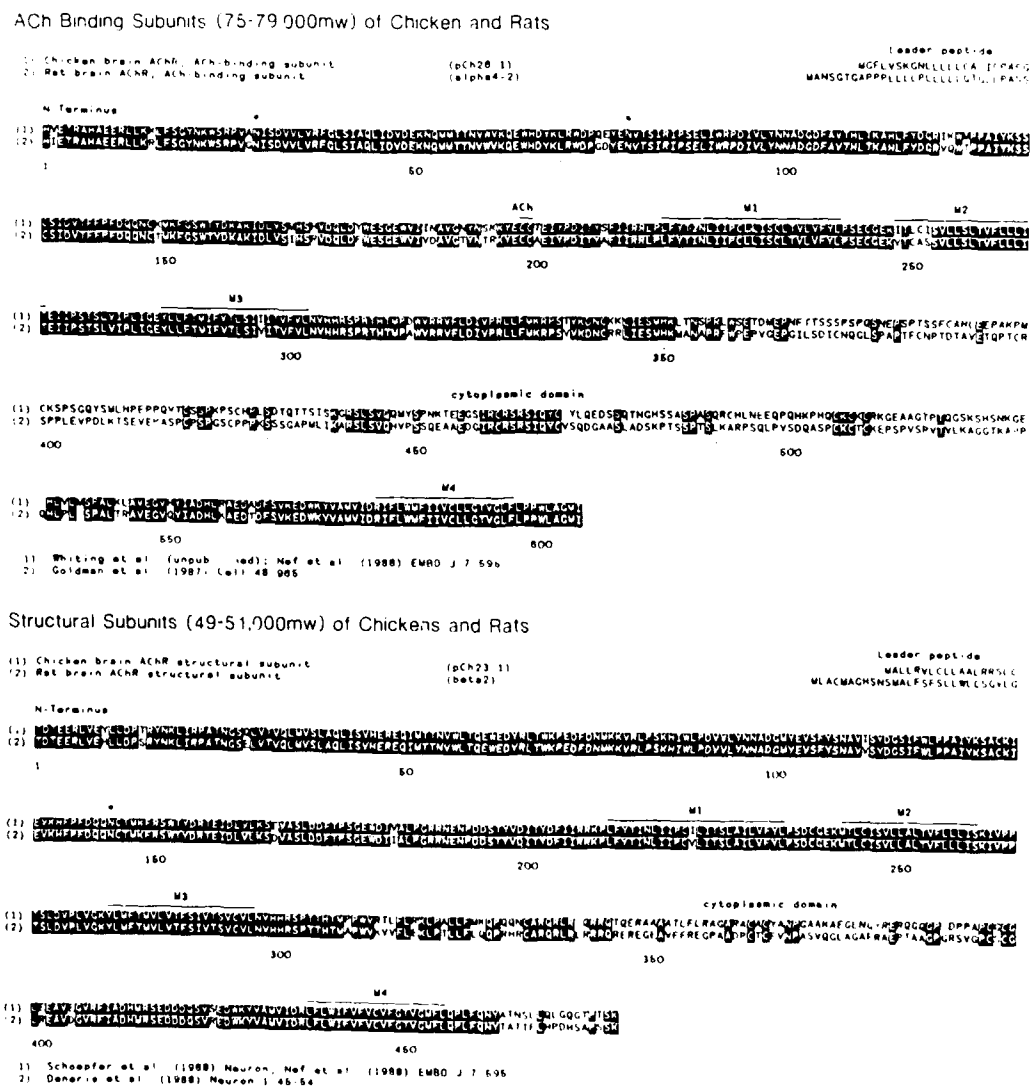


Figure 3. Comparison of the sequences of neuronal nicotinic receptors from chickens and rats.

subunits have been termed $\alpha 1$. The $\alpha 2$ cDNA[30,32] may, on the basis of our incomplete and unpublished N-terminal amino acid sequence data, correspond to the chicken brain acetylcholine-binding subunit of 59,000 apparent molecular weight.[20] The $\alpha 3$ cDNAs of chickens[26,32] and rats[27] probably correspond to the acetylcholine-binding subunit of ganglionic nicotinic receptors.[34] The $\alpha 4$ cDNA[29,32] corresponds to the chicken subtype with an acetylcholine-binding subunit of 75,000 apparent molecular weight[24] and to the predominant subtype purified from brains of mammals.[23] In mammalian brain,[35] $\alpha 2$, $\alpha 3$, $\alpha 5$, etc., appear to code for minor nicotinic receptor subtypes. Multiple structural subunit cDNAs have been recognized.[25,31,33] In rat brain the $\beta 2$ cDNA is most prevalent.[35] We have shown that different subtypes can use the same structural subunit.[20] Some subtypes may have unique structural subunits. There may also be developmental changes in subunit usage. For example, muscle nicotinic receptors before innervation or after denervation are composed of α, β, γ, and δ subunits, whereas at mature neuromuscular junctions receptors are composed of α, β, ϵ, and δ subunits.[36] A similar phenomenon might occur with neuronal nicotinic receptors.

In addition to neuronal nicotinic receptors, there are neuronal α-bungarotoxin-binding proteins.[2] There may be multiple subtypes.[37] Their function is unknown; they do not appear to be acetylcholine-gated ion channels.[38] Larger numbers of these proteins than nicotinic receptors are present, which may suggest that they are important. Their subunit structure is poorly characterized.[22,39] The pharmacological properties and a partial N-terminal amino acid sequence for one of the subunits suggests that α-bungarotoxin-binding protein belongs to the nicotinic receptor gene family.[39]

PROGRESS DURING THE PAST YEAR

Human Muscle Nicotinic Receptors in TE671 Cells

Sucrose gradient analysis of the receptors synthesized by TE671 cells revealed the presence of two components that bound ^{125}I-α-bungarotoxin (Figure 4). The larger of the two components, about 9S, sedimented at about the same rate as receptor monomers from Torpedo electric organ, as expected. The smaller component sedimented at about 5S. Although the 5S component bound α-bungarotoxin, it had very low affinity for small cholinergic ligands like carbamylcholine. The 5S component, like native receptor, also bound mAb 210, which is directed at the main immunogenic region (MIR) on α subunits. Thus, this component appears to exhibit the properties of a synthetic intermediate of α subunits previously recognized in pulse labeling experiments with the mouse muscle cell line BC3H-1 (Figure 5).[40-43] In that case, it was shown that three or four times as many α subunits are synthesized as are ultimately assembled into native receptors. The excess is rapidly destroyed. The nascent α subunits are cotranslationally glycosylated. The nascent BC3H-1 α subunits are recognized by mAb 61, which also recognizes denatured α subunits, but not by mAb 35, whose binding depends on the native conformation of the MIR. The nascent chains also do not bind α-bungarotoxin. During the next 30 minutes α subunits in a 5S form mature in conformation so that they can bind mAb 35 and α-bungarotoxin. High affinity for small cholinergic ligands is acquired only after assembly with the other subunits to form native receptor. In TE671 cells very large amounts of this synthetic intermediate are present, and it should be possible to characterize its biochemical properties in detail.

Torpedo Electric Organ Nicotinic Receptors Synthesized in Transformed Yeast

This is a collaborative project with the group of Dr. George Hess at Cornell University, who transformed the yeast with cloned cDNAs for the subunits of receptors from Torpedo electric organ.[44-47] His hope was to use this as a model for expressing large amounts of protein in yeast to permit study of receptor types which were not normally available in significant numbers. We studied the biochemical properties of these transformants.

First, a yeast strain was transformed only with Torpedo receptor α subunits.[44] Small amounts of subunits could be detected in Western blots. Their properties were studied in a sandwich radioimmunoassay. A mAb to α subunits (mAb 173) coated on plastic microwells was used to immobilize detergent-extracted α subunits, whose properties were then characterized by the binding of ^{125}I-labeled ligands (Figure 6). α subunits expressed in yeast were as effective as sodium dodecylsulfate (SDS)-denatured purified α subunits or native receptor in binding ^{125}I-mAb 142 (Figure 6A).

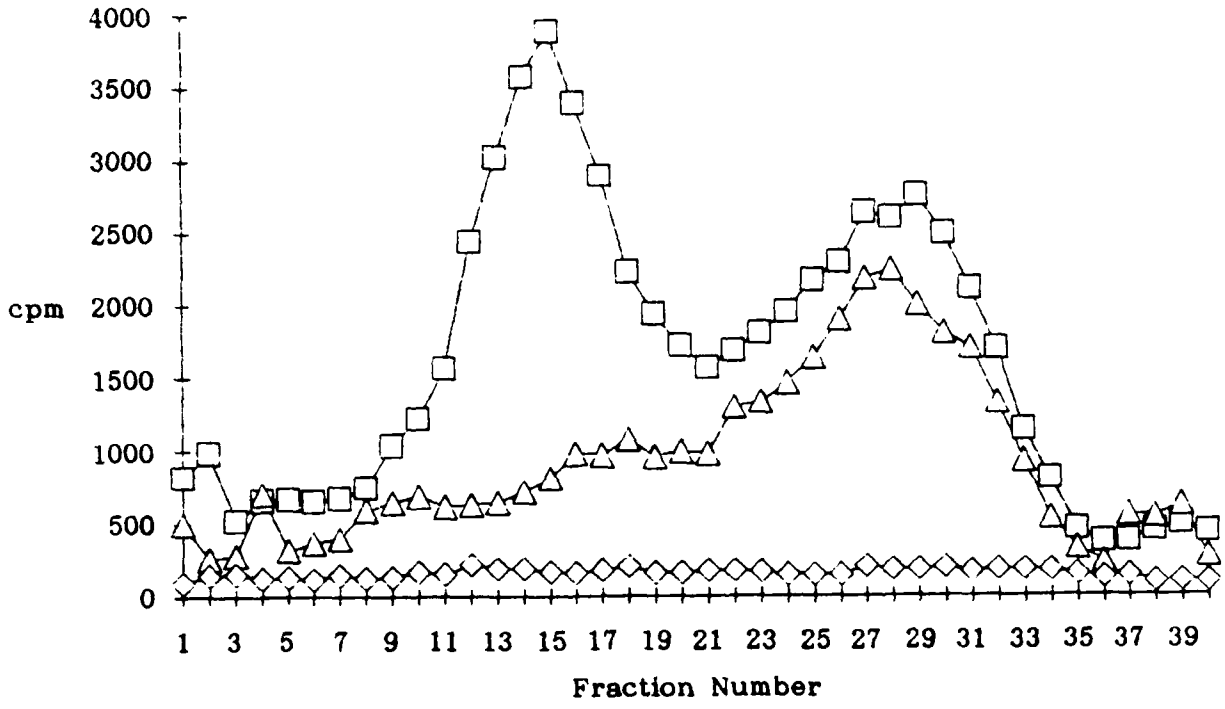


Figure 4. Sucrose gradient sedimentation analysis of receptors extracted from TE671 cells. *^{125}I-α-bungarotoxin-labeled receptors and unassembled α subunits were resolved by centrifugation on sucrose gradients in the absence of competing ligand ([]-[]) in the presence of 100 mM carbamylcholine (Δ-Δ), or in the presence of 1 μM unlabeled α-bungarotoxin as a specificity control. ^{125}I-α-bungarotoxin binding in each fraction was measured using a solid-phase assay in which mAb 210 (to the MIR) was used to anchor receptors to plastic microtiter wells. Monomers of receptors from Torpedo electric organ peak in fraction 17, and dimers peak in fraction 9. Note that carbamylcholine effectively competes for ^{125}I-α-bungarotoxin to native receptor, but has very low affinity for the unassembled α subunits, indicating that the conformation of the acetylcholine binding sites of the unassembled α subunits has only partially matured.*

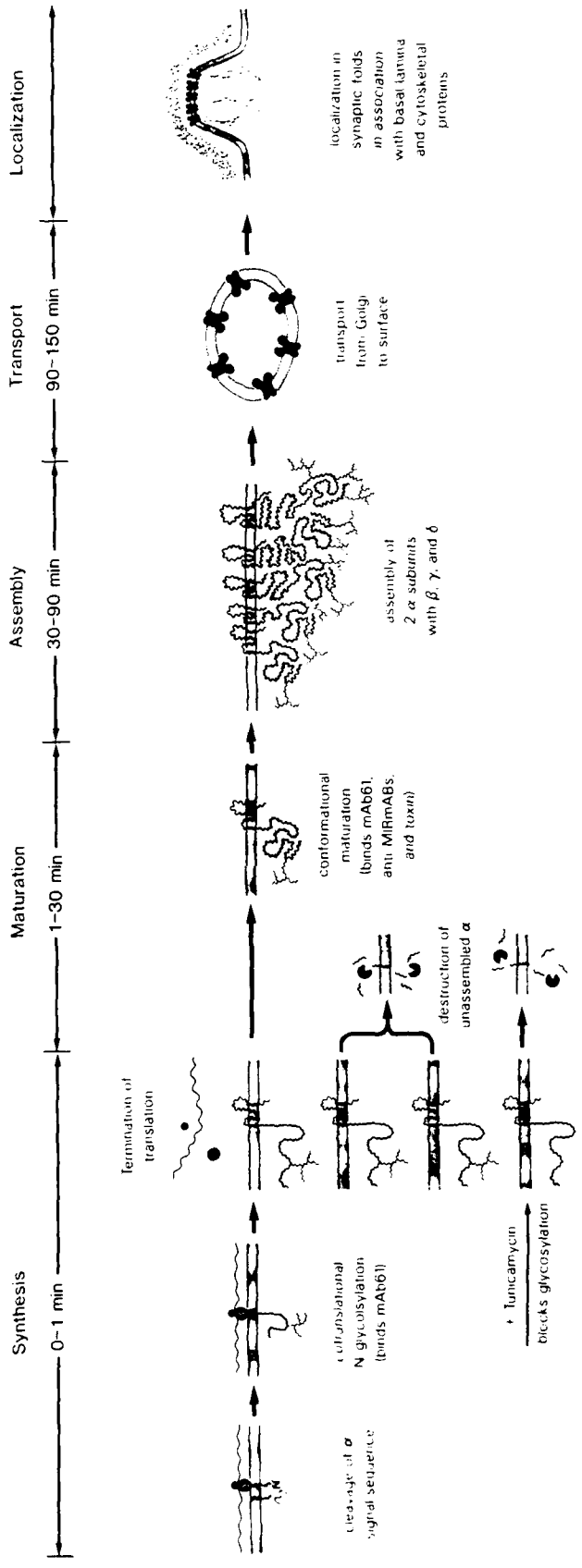


Figure 5. Assembly of receptor subunits in the mouse muscle cell line BC3H-1. This summary of results from pulse labeling experiments was reproduced from reference 41.

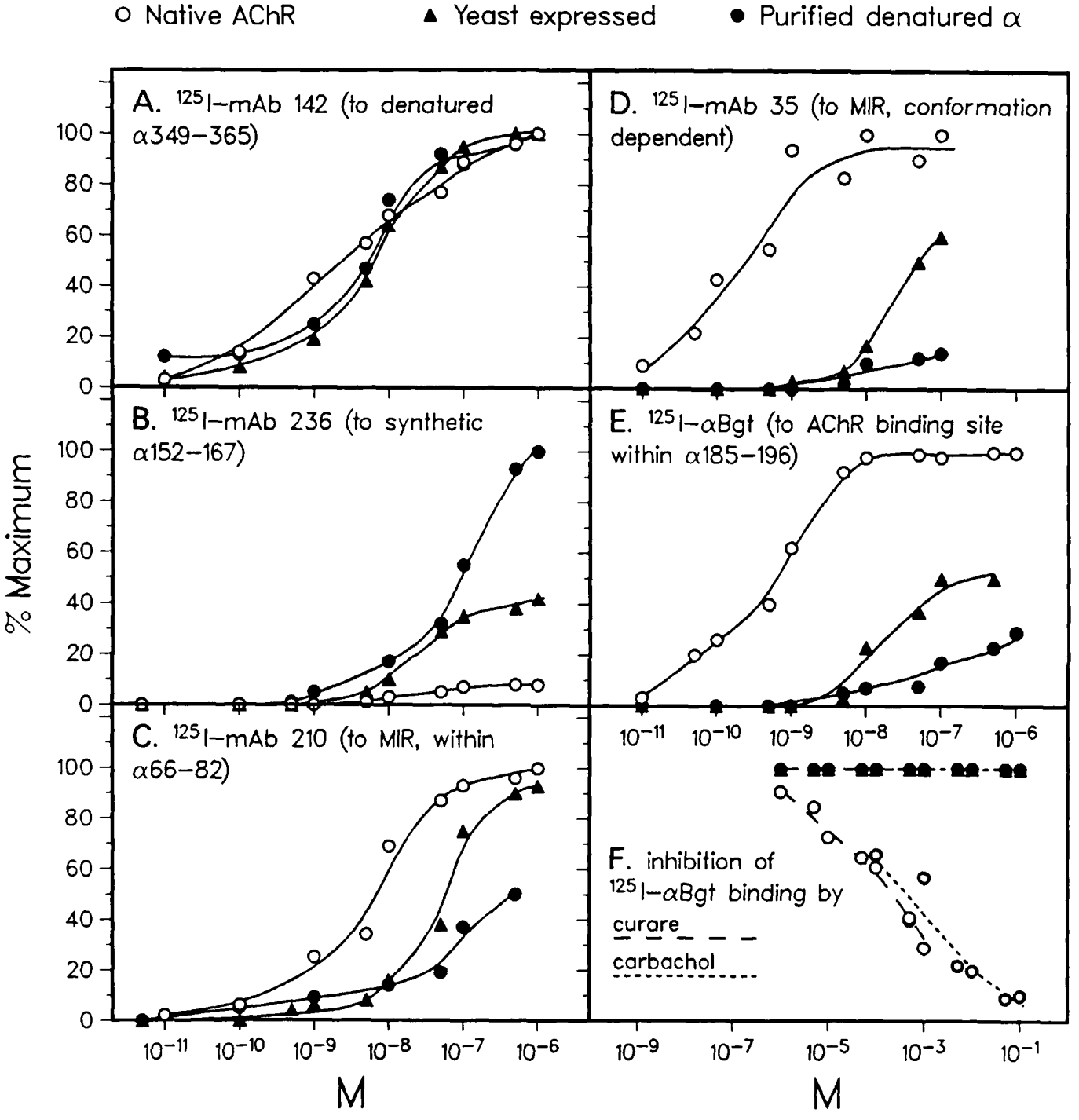


Figure 6. Properties of <u>Torpedo</u> nicotinic receptor α subunits expressed in yeast cells compared with SDS-denatured α subunits and native receptors by a sandwich radioimmunoassay. *Plastic microwells coated with mAb 173 were used to immobilize receptor or subunits and then binding of ^{125}I-labeled ligands was measured.*

This mAb binds to the sequence α349-365 on the cytoplasmic surface of α subunits.[9,48,49] This part of the receptor appears very flexible and can achieve similar conformations in both native and denatured receptor. α subunits expressed in yeast were intermediate in affinity for ^{125}I-mAb 236, between denatured α subunits (which had high affinity for this mAb) and native receptor (which had very low affinity) (Figure 6B). mAb 236 was made to the synthetic peptide α152-167 and basically cannot bind to α subunits in their native conformation,[9,48-50] but it detects an intermediate conformation in α subunits expressed in yeast. α subunits expressed in yeast bind ^{125}I-mAb 210 with an affinity intermediate between native receptors (which have high affinity) and denatured α subunits (which have low affinity) (Figure 6C). mAb 210 binds to the MIR of native receptor with high affinity[48,49] and binds with low affinity, but high specificity, to the synthetic α subunit peptide α66-76. α subunits expressed in yeast have much higher affinity for ^{125}I-mAb 35 than do denatured α subunits, but much lower affinity than do native receptors (Figure 6D). mAb 35 binds to the MIR of native receptor with high affinity, but has no detectable affinity for synthetic α subunit peptides.[13,48-51] The results with mAbs 210 and 35 suggest that α subunits in yeast exhibit a conformation of their MIR intermediate between denatured and native receptors. α subunits expressed in yeast bind ^{125}I-α-bungarotoxin more avidly than do denatured α subunits, but much less avidly than do native receptors (Figure 6E). Carbamylcholine and curare are effective at inhibiting ^{125}I-α-bungarotoxin binding to native receptor, but not to α subunits expressed in yeast or to denatured α subunits (Figure 6F). These results suggest that the acetylcholine binding site of α subunits expressed in yeast, like the MIR, is in a conformation intermediate between native and denatured. Thus, when all are compared solubilized with nondenaturing detergents, the properties of the expressed α subunits closely resemble those of the intermediates of receptor synthesis detected in BC3H-1 cells and the unassembled α subunits detected in TE671 cells.

A yeast strain was transformed simultaneously with cDNAs for α, β, γ, and δ subunits of receptors from Torpedo electric organ.[47] mRNA extracted from this strain was injected into Xenopus oocytes and shown to code for the synthesis of functional receptors.[47] Thus, transcription of Torpedo receptor subunits in the yeast cells was effective. However, only small amounts of subunit proteins could be detected by Western blots, and no β subunit protein was detected. It was found that by making a chimera substituting most of the α subunit signal sequence for the β signal sequence, and by growing the yeast at 5°C, detectable amounts of β subunits could be made (Figure 7).[47] In any case, the α subunits retained the properties of the synthetic intermediate and did not assemble efficiently with the other subunits to form native receptors. There is clearly a translation or posttranslation problem in yeast which impairs their ability to synthesize large amounts of subunits and properly assemble them into native nicotinic receptors. One problem may be proteolysis of these foreign proteins in the yeast. Another problem may be different properties of the yeast

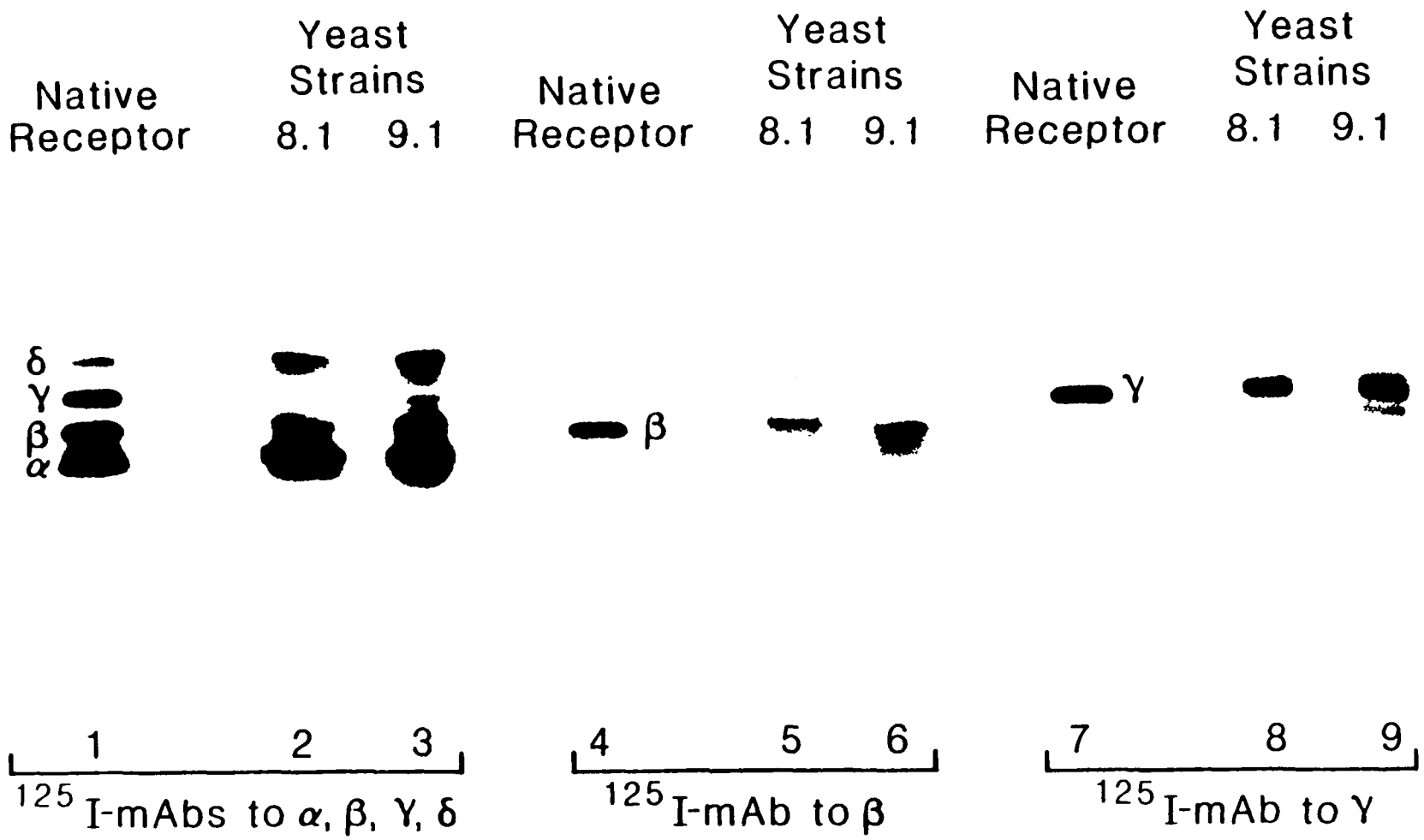


Figure 7. Yeast strains KUJ 8.1 and KUJ 9.1 transformed with Torpedo nicotinic receptor α, γ, and δ subunits and chimeras of most of the α signal sequence with β subunits express receptor subunits when grown at 5°C. Western blots were probed with 1 nM ^{125}I-mAb 142 to α subunits, 3 nM ^{125}I-mAb 111 to β subunits, 5 nM ^{125}I-mAb 168 to γ subunits, and 5 nM ^{125}I-mAb 166 to δ subunits. Autoradiograms were exposed 4-10 hours. Reproduced from reference 47.

membranes. For example, native receptors and α subunits can be easily solubilized from Torpedo electric organ, BC3H-1 cells, or TE671 cells, etc., using 0.5% Triton X-100 (Boehringer Mannheim, Indianapolis, IN). However, solubilization of receptor subunits from yeast required a mixture of detergents: 0.5% Zwittergent 3-14 (Calbiochem, San Diego, CA), 2% Triton X-100, and 0.05% SDS (Sigma, St. Louis, MO). A third problem could be incompatible "chaperonins"[52] required to assist assembly of a multisubunit protein like receptors. Whatever the reason, so far, yeast have not proven to be an effective system for synthesizing receptors from their cDNAs.

Mapping the MIR Using Chemically Synthesized α Subunit Peptides

The MIR is a highly conformation-dependent epitope on the extracellular surface of α subunits which is recognized by mAbs with high affinity only in the native receptor.[13,48-51] In 1986 we were able to use large proteolytic fragments of the α subunits of receptors from Torpedo to map the MIR to within the sequence α46-127.[48] Neither immunoprecipitation of smaller soluble synthetic peptides[51] nor reaction with synthetic octomers synthesized coupled to plastic pins by the Geysen method[9] were effective in our hands at detecting the low affinity of anti-MIR mAbs for synthetic peptides. Recently Tzartos[53] and coworkers were able to detect binding of anti-MIR mAbs to shorter peptides by using an assay in which the peptides were bound to polylysine-coated plastic microwells. We were able to confirm and extend these results, as shown in Figure 8 and summarized in Figure 9. Many mAbs to the MIR bind to the peptide α68-76. Thus, some of the amino acids which form the MIR are in this sequence. Some mAbs, such as mAb 35, appear to be absolutely conformation dependent. Presumably these mAbs recognize amino acids which are contiguous only in the native conformation of the receptor, whereas those mAbs which bind to synthetic peptides probably recognize several consecutive amino acids in the peptide as part of their epitope. Curiously, mAbs to the MIR bind to muscle nicotinic receptors from essentially all species tested, except Xenopus.[54] The mAbs tested also do not bind to the Xenopus sequence α66-76. This establishes the biological significance of the binding assay and suggests that asparagine α68 and aspartate α71 are important contact residues. In the Xenopus[55] peptide α68 is aspartate and α71 is a lysine, altogether nonconservative changes. Removing α68 converts the human sequence[18,19] α68-76 from one which binds mAb 210, to α69-76, which does not, further demonstrating the importance of asparagine α68. These results permit us to map this important epitope with fairly high resolution. The function of this part of α subunits is unknown; mAbs bound to it do not interfere with ligand binding or channel function.[56,57] It is a pathologically important epitope in myasthenia gravis.[1] The highly conserved structure of the MIR suggests that it may be important for an aspect of receptor function yet to be appreciated.

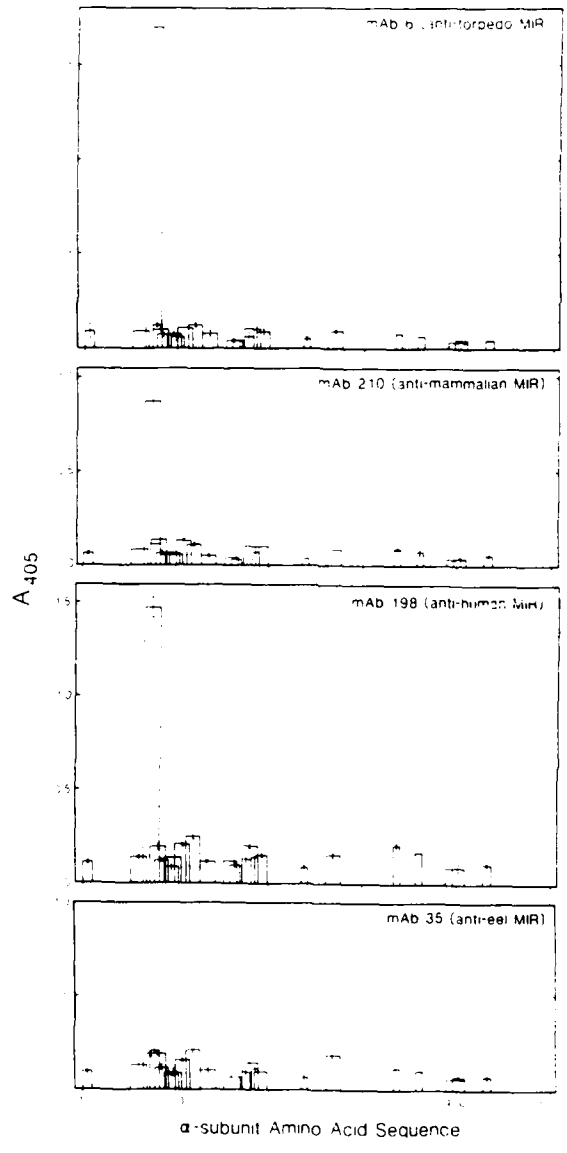


Figure 8. Mapping the MIR epitope on the sequence of α subunits by an enzyme-linked immunoassay using synthetic <u>Torpedo</u> acetylcholine receptor α subunit peptides coupled to polylysine-coated microwells to detect binding of mAbs to the MIR. *Peptides were glutaraldehyde coupled to polylysine-coated wells. Phosphate-buffered saline containing 1% bovine serum albumin, 1% ovalbumin, and 0.1% Tween 20 to inhibit nonspecific binding was used to quench and wash the wells and for all subsequent incubations. mAbs at concentrations >1 μM were allowed to bind overnight. After three washes bound mAbs were detected using an anti-rat IgG mAb coupled to peroxidase.*

Synthetic peptides that *bind* to MIR mAbs:

α	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	
	R	W	N	P	A	D	Y	G	G	I	K	K	I	R	L	P	S	(tyr 83) *Torpedo* α66-83
	K	W	N	P	D	D	Y	G	G	V	K							Human α66-76
			N	P	D	D	Y	G	G	V	K							Human α68-76

Synthetic peptides that *do not bind* to MIR mAbs:

α	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	
	K	W	D	P	A	K	Y	G	G	V	K							*Xenopus* α66-76
				P	D	D	Y	G	G	V	K							Human α69-76
					D	D	Y	G	G	V	K							" α70-76
						D	Y	G	G	V	K							" α71-76
		W	N	P	D	D			could these have failed									" α67-7[illegible]
		W	N	P	D	D	Y		for lack of K to glutar-									" α67-72
			N	P	D	D	Y	G	aldehyde link in ELISA?									" α68-73
...																		*Torpedo* α44-60
								G	G	I	K	K	I	R	L	P	S ...	" α73-90
													I	R	L	P	S ...	" α78-93

" α1-11
" α44-60
" α89-104
" α100-116
" α112-127
" α127-143
" α159-170
" α172-188
" α185-199
" α172-205
" α194-212
" α261-277
.
.
.
~*Torpedo* α330-437

Figure 9. Summary of the reaction of synthetic peptides with mAb 210 using the enzyme-linked immunoassay described in Figure 8.

Subunit Stoichiometry of Neuronal Nicotinic Receptors

Previously, we had shown that neuronal nicotinic receptors were composed of only two kinds of subunits.[8,20,22,23] Further, we showed that there were at least two copies of each subunit present in each receptor by showing that a receptor bound to a subunit-specific mAb coupled to an agarose bead could still bind another ^{125}I-labeled mAb of the same type.[20,23]

Now we have developed a technique for determining the relative number of subunits in a receptor.[58] Purified receptor is denatured in a mixture of 4 M urea and 1% SDS to make all of its tyrosines equally accessible to labeling. After labeling with ^{125}I, the subunits are separated by electrophoresis on acrylamide gels in SDS, located by brief contact autoradiography, and then the subunit bands are cut out of the gel and quantitated by gamma counting. The tyrosine content of each subunit known from the cDNA sequence is used to correct slightly for the difference in tyrosine content in each subunit, and then the relative amounts of the subunits are compared. Figure 10 shows that this method yields the expected stoichiometry[15,59,60] for receptor from Torpedo, $\alpha_2\beta\gamma\delta$. Further, it shows that in neuronal nicotinic receptors from chicken, rats, and cattle, there are equal numbers of acetylcholine-binding and structural subunits. This proves that neuronal nicotinic receptors cannot have the pentagonal symmetry[13,14] of muscle nicotinic receptors.

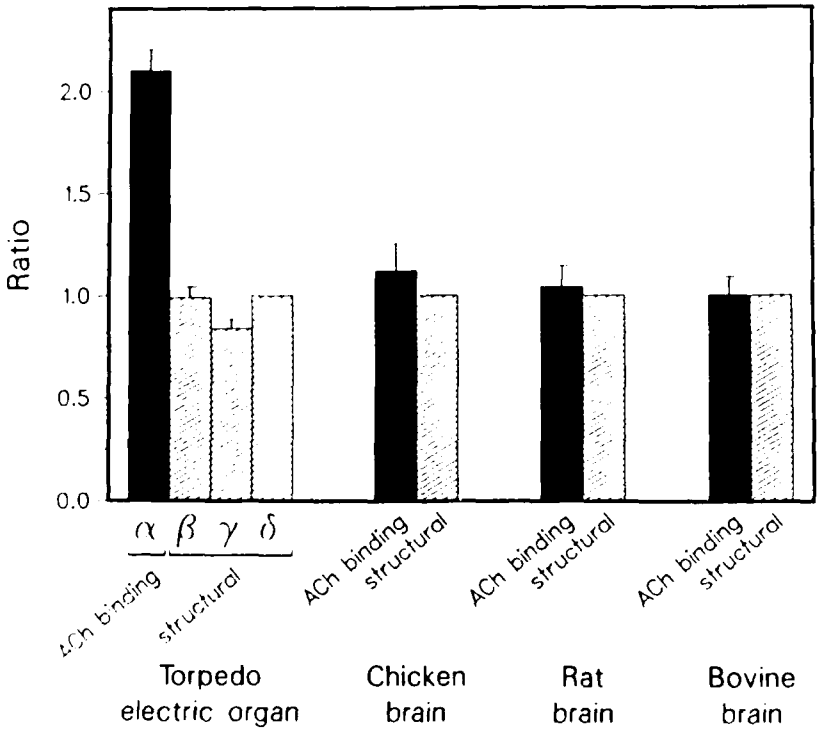


Figure 10. Ratio of subunits in Torpedo electric organ nicotinic receptors and receptors from brains of chickens, rats, and cattle determined by labeling with ^{125}I and determining the relative amount of ^{125}I in each subunit. *Reproduced from reference 58.*

Knowing that neuronal nicotinic receptors are composed of equal numbers of two kinds of subunits and at least two copies of each subunit, we then tried to determine the number of copies by determining the molecular weight of the receptor. Sucrose gradient sedimentation revealed apparent molecular weights for neuronal receptors similar to those of Torpedo receptor monomers (Figure 11). The protein molecular weight of Torpedo nicotinic receptor monomers calculated from the subunit composition $\alpha_2\beta\gamma\delta$ and the calculated subunit molecular weights[16] α=50,116; β=53,681; γ=56,279; and δ=57,565 is 267,757. With the addition of two ^{125}I-α-bungarotoxin molecules, the protein molecular weight becomes 283,757. Sugars[61] add an additional 19,609 for a total of 303,366. Thus, the apparent molecular weight (determined by sucrose gradients) of rat brain nicotinic receptors of the α4 subtype is about that of Torpedo receptor monomers, whose calculated molecular weight is about 303,000.

The molecular weights of neuronal nicotinic receptors composed of two subunits of each kind were calculated in the same way. The deduced molecular weights of the rat brain α4 receptor subtype structural[31] and acetylcholine-binding subunits[29] are 54,484 and 67,122, respectively. Thus, the protein molecular weight of a receptor composed of two subunits of each type is 243,212. In Torpedo receptor, eight of the ten potential N-glycosylation sites appear to be used, with an average of 2451 molecular weight for each of the 1.6 glycosylation sites per subunit.[61] If the two potential N-glycosylation sites on the structural subunits and the two on the acetylcholine-binding subunits of the rat brain receptor had sugar substituents of this size, the total molecular weight would be 262,820. Similarly, the molecular weight for a chicken brain receptor composed of two structural subunits[25,32] of 54,000 and two α4 acetylcholine-binding subunits[26,32] of 68,400 deduced protein molecular weight, and 4902 contributed by sugars, would be 264,408. The molecular weight of a chicken brain receptor of the other subtype in extracts with two structural subunits of 54,000 deduced molecular weight and two α2 acetylcholine-binding subunits[32] of 58,090 deduced protein molecular weight, and similar glycosylation, would be 243,788.

We compared the calculated molecular weights of neuronal nicotinic receptors with their sedimentation behavior on sucrose gradients. When solubilized in Triton X-100, the two chicken brain receptor subtypes are not resolved by sucrose gradient centrifugation. This is not surprising, considering that they may differ in molecular weight by only 20,620. It is more interesting that both rat and chicken neuronal receptors solubilized in Triton X-100 sediment more rapidly than Torpedo receptor monomers, which have a deduced molecular weight 40,000-59,000 larger. Neuronal receptor composed of only four subunits might be more compact and less slowed by friction than five-subunit receptors from electric organs. An additional factor to consider is that α4 acetylcholine-binding subunits[26,29,32] contain a long, unique sequence in a part of the molecule known

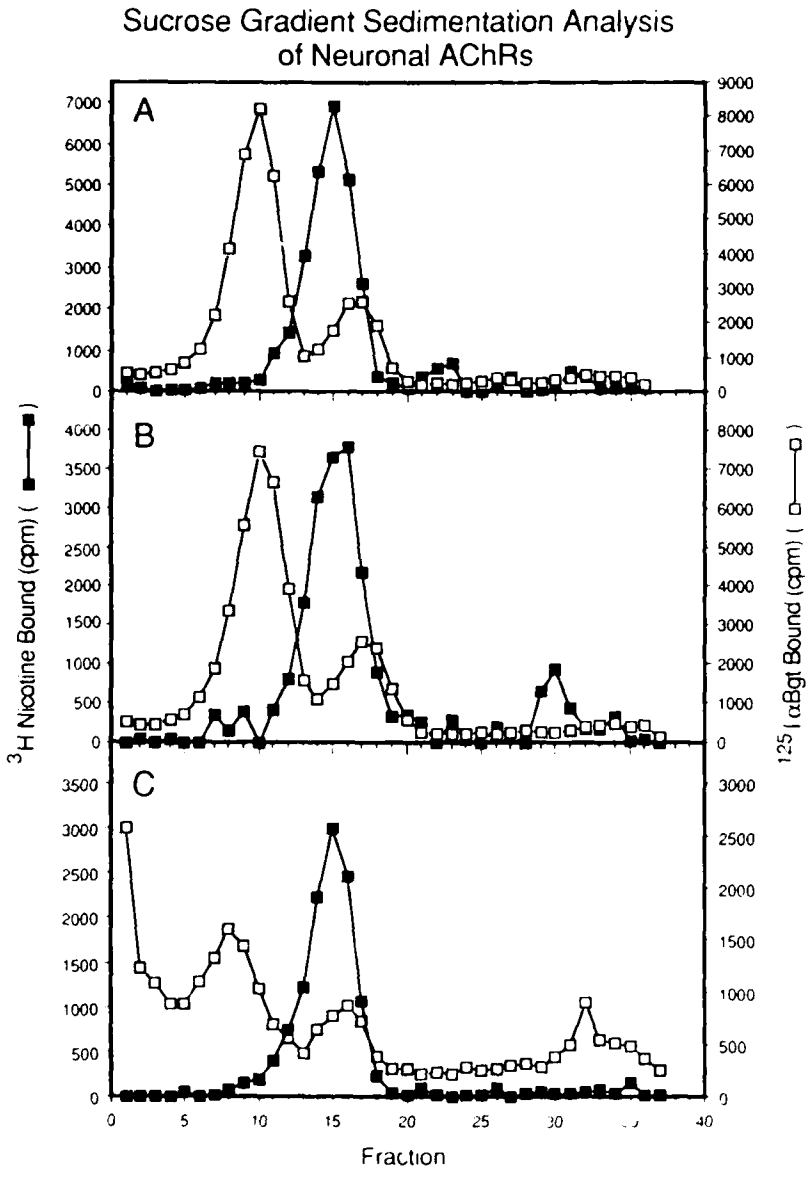


Figure 11. Sucrose gradient sedimentation analysis of neuronal nicotinic receptors. *Rat and chicken brain receptors were sedimented on 5-20% sucrose gradients as described in Methods. Each figure shown is the average of duplicate gradients. Fractions are numbered from the bottom of the gradients. Torpedo electric organ receptors trace labeled with ^{125}I-α-bungarotoxin were included in each gradient as an internal control (□--□). Neuronal receptors were quantitated by [^{3}H]-L-nicotine binding in an immunoimmobilization assay using mAb 270 coupled to agarose to bind the receptors through their structural subunits (■--■). Nonspecific binding has been subtracted from each immunoassay point. A) Rat brain and Torpedo receptors extracted with 2% Triton X-100. B) Chicken brain and Torpedo receptors extracted with 2% Triton X-100. C) Rat brain and Torpedo receptors extracted with 2% cholate/0.2% asolectin and sedimented on gradients containing 2% cholate/0.2% asolectin. Reproduced from reference 58.*

to be on the cytoplasmic surface of Torpedo receptor α subunits.[48,49] Antisera to this putative cytoplasmic sequence of chicken α4 subunits expressed in bacteria react well with both denatured subunits on blots and with native receptor,[26] suggesting that this part of the subunit exists in the same loosely constrained conformation in both native and denatured receptors. Thus, it is possible that this extended putative cytoplasmic domain might significantly alter the shape of the neuronal receptor with respect to receptors from Torpedo and cause detectable variance in sedimentation behavior.

Yet another possible explanation for the faster sedimentation of neuronal receptors is that neuronal receptors solubilized from lipid-rich brain might have additional mass due to lipid not displaced by Triton X-100. Solubilizing in a cholate-lipid mixture did not alter the sedimentation of rat brain receptors, but increased the rate of sedimentation of receptor monomers and dimers from Torpedo, resulting in cosedimentation of rat brain receptors and Torpedo receptor monomers. This shows that solubilization conditions can affect apparent molecular weight and that sucrose gradient sedimentation is not sufficiently resolutive to precisely determine the molecular weight of receptors. If there were three copies of each rat brain receptor subunit, and similar assumptions, the molecular weight would be 394,230. The difference between the observed value of 303,000 for the apparent molecular weight and the calculated molecular weight for a tetrameric receptor composed of two subunits of each kind is 40,546. This difference is considerably less than 90,864, which is the difference between the observed value and the calculated value for a hexameric receptor with three subunits of each kind.

Three additional factors argue in favor of the idea that neuronal nicotinic receptors are composed of two subunits of each kind rather than three of each kind. First, chicken brain receptors expressed in Xenopus oocytes exhibit a conductance of 20pS, approximately half of the values obtained for electric organ and muscle receptors expressed in oocytes.[62] A channel formed by four subunits rather than five might be expected to be smaller and exhibit lower conductance, whereas a channel formed by six subunits might be expected to exhibit a larger conductance. Second, chicken brain receptors expressed in oocytes exhibit a Hill coefficient for activation by acetylcholine of 1.5, a value similar to those observed for electric organ and muscle receptors expressed in oocytes (1.6-1.7).[62] Electric organ receptors are known to have two acetylcholine-binding subunits, as would be found in a tetrameric neuronal receptor, whereas a hexameric receptor with three acetylcholine-binding subunits might be expected to have a Hill coefficient closer to 3. A third argument in favor of neuronal nicotinic receptors being composed of just two subunits of each kind rather than three of each kind is that the tetrameric arrangement is the simplest assembly possible consistent with the observations of more than one copy of each subunit, equal numbers of each

subunit, and a molecular weight of about 303,000. Biosynthesis of a unique assembly of hexamers of two kinds of subunits without also forming some tetramers, octomers, etc., might be a problem.

Figure 12 compares, in diagrammatic form, the pentagonal arrangement[13,14] of subunits known to occur in receptors of Torpedo electric organ with other subunit arrangements. Pentagonal arrangements of two kinds of subunits ($\alpha_2\beta_3$) are inconsistent with the observation that neuronal receptors have equal numbers of both subunit types, and would require one subunit to bind specifically to both identical and different subunits through the same interface. Hexagonal arrangements of three subunits of each kind ($\alpha_3\beta_3$) are consistent with the requirement of neuronal receptors for equal numbers of each subunit, but lack the simplicity of the tetrameric arrangement, and might have larger molecular weights, larger channels, and larger Hill coefficients than are observed. The favored tetrameric arrangement for neuronal receptors of two subunits of each kind is depicted at the bottom of the figure. This model is consistent with the requirement for equal numbers of two subunits and might exhibit the same Hill coefficient, but smaller conductance, than the pentameric muscle-type receptor. Further, biosynthesis of a unique assembly of two subunits of each kind to form a central channel might be facilitated by specific interaction at unique interfaces between dissimilar subunits to form a closed array. This arrangement preserves the feature observed in electric organ receptor[13] of two acetylcholine-binding subunits separated by subunits which contribute to the structure of the channel.

Structure and Developmental Expression of Neuronal Nicotinic Receptors Studied Using cDNAs and mAbs

Previously, we reported the sequences of cDNAs for the structural subunit and α4 acetylcholine-binding subunit of receptors from chicken brains.[25,26] We have also succeeded[26] in cloning a partial cDNA for the ganglionic-type acetylcholine-binding subunit termed α3 by screening with a cDNA for the rat α3 subunit obtained from Drs. Patrick and Heinemann.[27] Its sequence is compared with that of the other two subunits in Figure 13. Its sequence is virtually identical to a chicken cDNA reported by Ballivet and coworkers.[32] An internal Eco RI restriction site terminates this clone and others we obtained for α3 short of the expected N-terminus.

We investigated the developmental expression of neuronal nicotinic receptors in chickens at the levels of both RNA and protein (Figure 14).[26] In brain, mRNA for the α4 acetylcholine-binding subunit increased steadily until embryonic day 20, then decreased in the adult. The amount of mRNA for the structural subunit plateaued at embryonic day 11, then dramatically decreased in the adult brain. mRNA for α3 was undetectable in brain. In retina, α4 mRNA reached a maximum by embryonic day 14, and decreased thereafter. The structural subunit mRNA occurred as two species whose relative amounts changed during development. The α3 mRNA was vastly more abundant in retina than in brain.

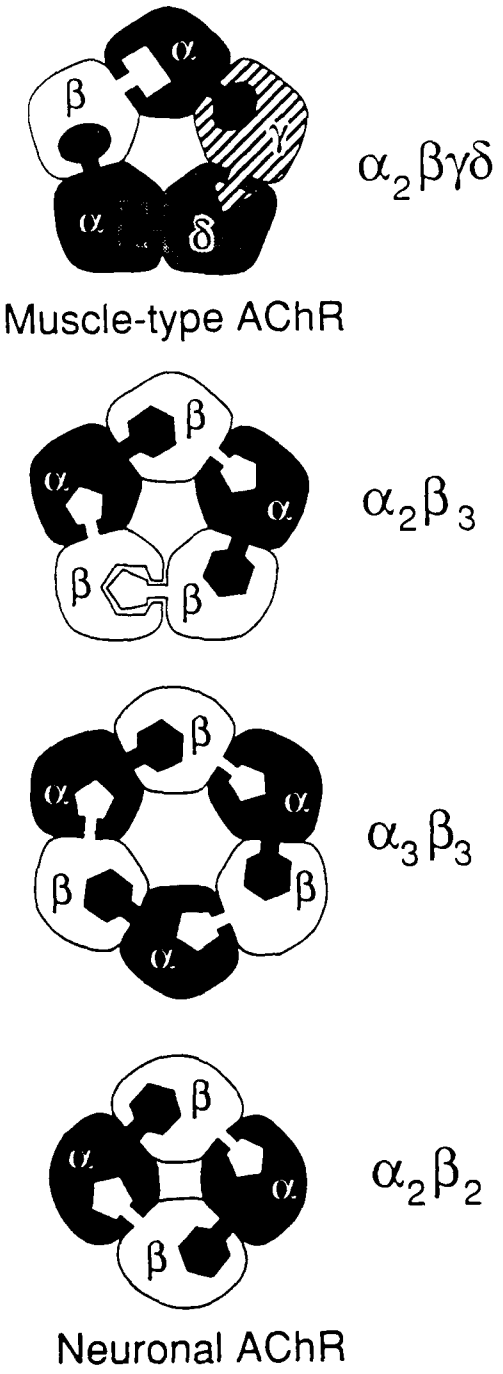


Figure 12. Nicotinic receptor subunit organization. *Diagrammatic end-on views of subunits around the cation channel are shown. The interlocking contacts between each subunit depict conserved (probably hydrophobic) interfaces through which the subunits might assemble to form the channel. Reproduced from reference 58.*

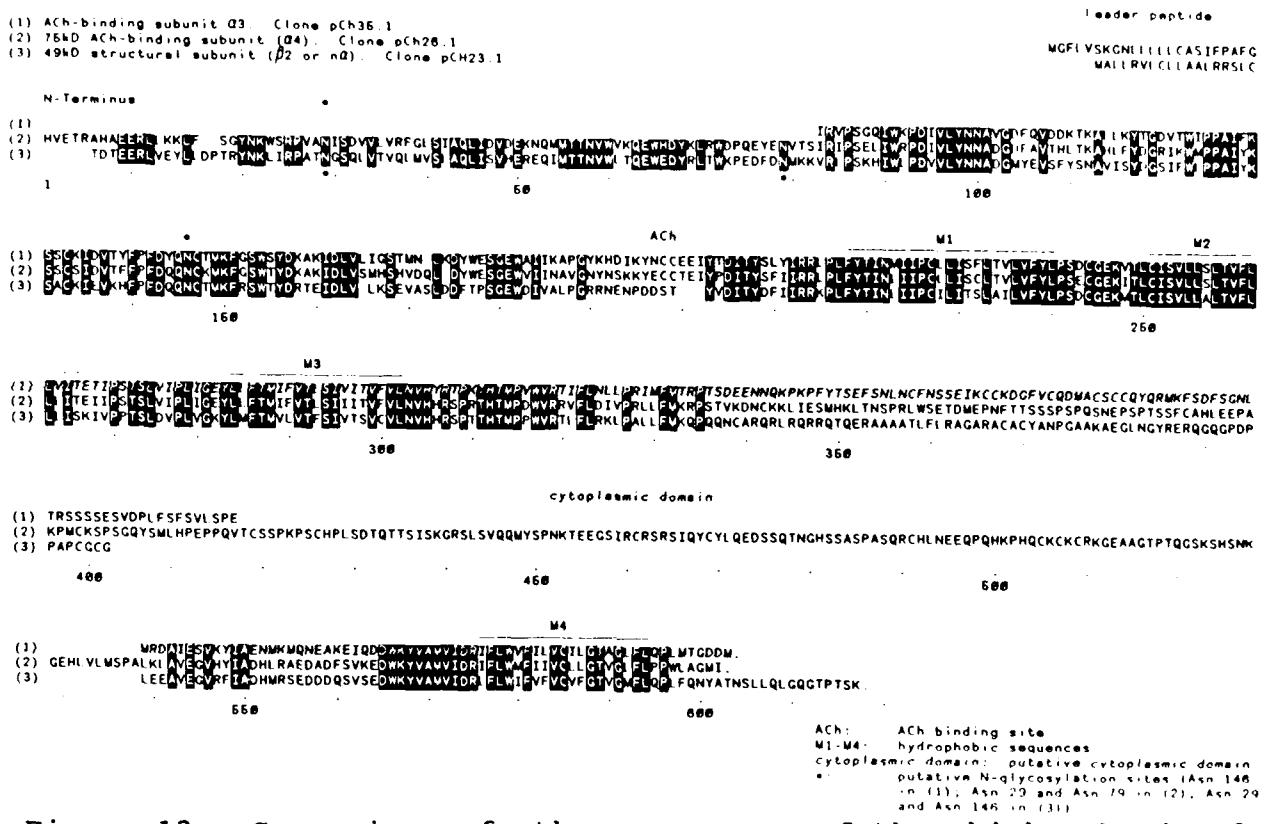
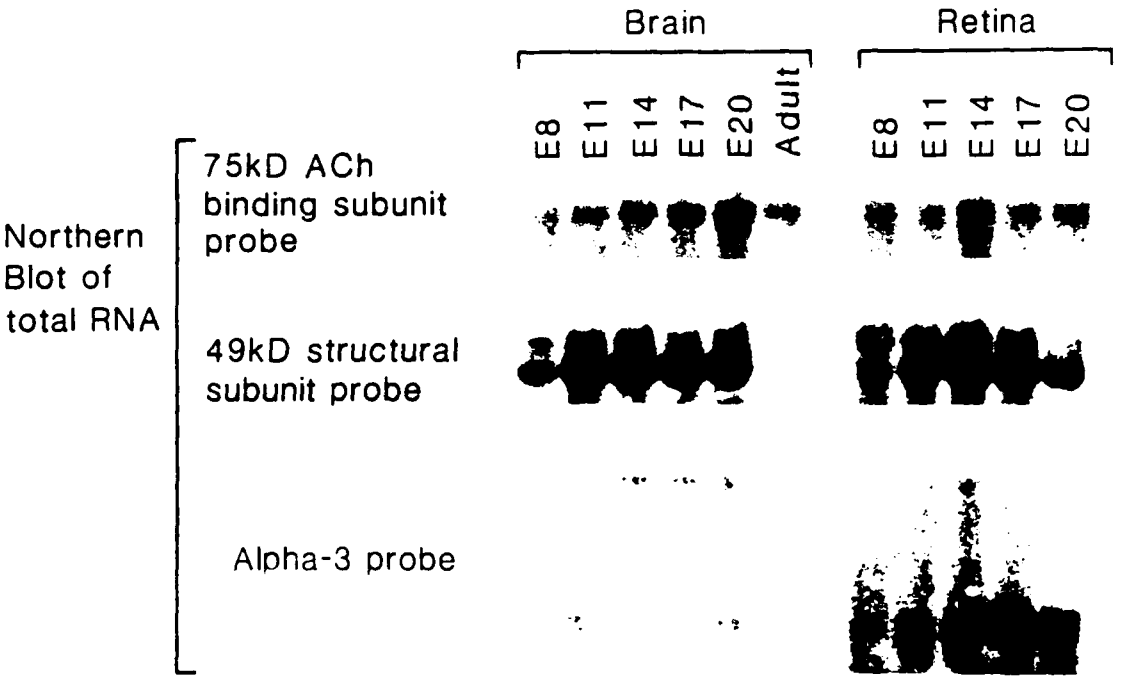


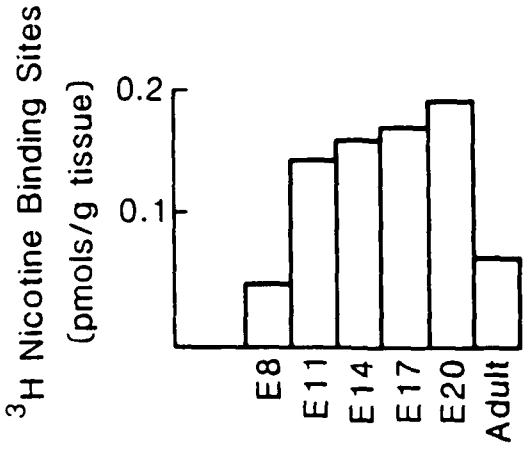
Figure 13. Comparison of the sequences of the chicken brain α3 acetylcholine-binding subunit (a partial clone), with the sequences of the α4 acetylcholine-binding subunit and the structural subunit. *Reproduced from reference 26.*

Measurement of high-affinity nicotine binding sites during development (Figure 14) would probably reflect only α4- and perhaps α2-type receptors, since ganglionic α3-type receptors have much lower affinity for nicotine, but the negligible level of α3 mRNA in brain suggests that there is very little if any of this protein in brain extracts. The amount of nicotine binding in brain extracts parallels the amount of α4 mRNA. The relative amounts of the α4 subtype and the other brain subtype with high affinity for nicotine (α2?) were radioimmunoassayed as previously reported and found to change during development, reaching nearly equal amounts by embryonic day 17.

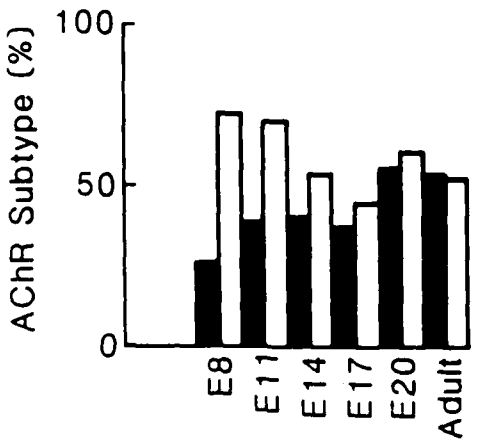
To get an immunogen for raising subunit-specific antisera whose binding might be more resistant to histological fixatives than is the binding of most mAbs, we expressed a unique fragment of chicken α4 subunits in bacteria.[26] In Torpedo α subunits we have shown that the sequence between the third and fourth hydrophobic domains is on the cytoplasmic surface, is relatively immunogenic, and is antigenic in both native and denatured receptors.[48,49] The corresponding part of neuronal nicotinic receptors is unique to each subunit. Therefore, this region was chosen for bacterial expression. A fragment corresponding to α4 methionine 335-alanine 517 was subcloned into the heat-inducible

Figure 14. Expression of nicotinic receptors in developing chicken brain and retina.





Total ^{3}H nicotine binding sites in brain detergent solubilized membranes.



Proportion of AChR subtypes in brain detergent solubilized membranes.

■ Subtype with 59kD ACh binding subunit

□ Subtype with 75kD ACh binding subunit

Figure 14. Expression of nicotinic receptors in developing chicken brain and retina. *The 75kD acetylcholine-binding subunit is coded for by the cDNA α4. (1) Northern blot analysis: Total brain and retina RNA (20 μg/lane) was resolved through a formaldehyde-containing agarose gel, transferred to a nylon membrane, and then hybridized at high stringency with each ^{32}P-labeled cDNA probe. Only the relevant part of each blot is shown. (2) Total ^{3}H-nicotine binding sites in detergent extracts of brain were determined by a glass fiber filter binding assay. Detergent extracts were prepared from 4-8 g of brain tissue for each time point, as previously described. Quadruplicate aliquots (200 μl) of detergent extract were incubated for 1 hour, 4°C, with 20 nM ^{3}H-nicotine, and then diluted with 4 ml of ice-cold 50 mM Tris, pH 7.6, and filtered through Whatman GF/B filters presoaked in 0.3% polyethylenimine. Filters were washed 3 times with 4 ml of the same buffer and bound radioactivity determined by scintillation counting. Nonspecific binding was determined by incubation in the presence of 1 mM nonradioactive nicotine, and has been subtracted. (3) The proportion of receptor subtypes was determined by shaking aliquots of each detergent extract with mAb 35-Sepharose (to deplete receptors with the 59kD acetylcholine-binding subunit), or mAb 285-Sepharose (to deplete receptors with the 75kD acetylcholine-binding subunit), and goat anti-rat IgG Sepharose (which will not deplete any receptors and therefore is a control to determine total receptors) overnight, 4°C. The Sepharose was pelleted and the ^{3}H-nicotine binding sites in the extracts measured by filter binding assay, as described above. The percent of the total ^{3}H-nicotine binding sites depleted by each mAb (i.e. the percent of that receptor subtype) was determined and expressed as a percent of the total receptors. "E8," "E11," etc., refer to embryonic day 8, 11, etc. Reproduced from reference 26.*

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bacterial expression vector pJLA602. Electrophoresis of transformants grown at 42°C revealed no unique band by Coomassie staining, indicating that only small amounts of the protein were expressed. However, Western blots with mAb 289 (which is specific for this subunit)[20] revealed an inducible protein of apparent molecular weight 24,000, quite close to the calculated molecular weight of 22,900 for the expressed fragment. Using an affinity column of mAb 289, the recombinant protein was purified. Immunization of rats produced a subunit-specific antiserum with a titer of 0.6 μM.

To be able to more efficiently express large numbers of subunit-specific fragments of other cDNAs for use as immunogens, we investigated another bacterial expression system. This was first applied to the chicken α3 clone large cytoplasmic loop α leucine 323-methionine 571. We used a system described by Rosenberg and Studier[63,64] in which a phage T7 polymerase gene driven by a lac promoter is integrated into the Escherichia coli strain BL21 (DE3), and the DNA fragment to be expressed is cloned into a plasmid behind a T7 promoter followed by a ribosome

binding site. The α3 fragment was cloned into a pET3c-derived vector to give an expressed protein with 15 N-terminal amino acids coded by the vector. Induced cultures produced copious amounts (perhaps 25% of total protein) of an apparent 17,000 molecular weight protein. The protein was in inclusion bodies which could be partially purified by differential centrifugation, and then finally purified by extracting contaminants successively with 3 M NaSCN and 0.5 M urea. The final material was dissolved in 8 M urea in phosphate-buffered saline containing 1 mM dithiothreitol. Removal of the urea by dialysis resulted in partial precipitation. This is now being used as an immunogen in rats and rabbits. Using this system, we have also expressed large amounts of several other subunit fragments, and these are in various stages of purification and immunization. This seems to be a very efficient system. Antisera will be adsorbed with induced empty vector extracts. Subunit-specific antisera from several species should allow us to localize simultaneously more than one type of receptor on the same tissue section. This should prove very useful for elucidating the functional roles of these receptors.

Histological Studies of Nicotinic Receptors

Nicotinic receptors were localized autoradiographically in zebra finch brains in a collaborative study with Dr. Thomas Podleski and coworkers at Cornell University, for which we provided the labeled reagents ^{125}I-α-bungarotoxin, ^{125}I-mAb 270, and ^{125}I-mAb 35.[65] These agents were localized throughout brains in substantial detail using frozen sections of unfixed tissue (Figure 15). mAb 270 to chicken brain nicotinic receptors binds to the structural subunits common to both receptor subtypes we purified from chicken brains.[20] Previously we used this mAb to purify and localize receptors in rat brains.[22] mAb 35 binds to only one of the two subtypes of receptors in chicken brains.[20] It is directed at the MIR and also binds to receptors in muscle.[13] It does not bind to receptors from rat brain. Like mAb 270, it binds to structural subunits of receptors from chicken brains. The binding pattern for mAb 270 and mAb 35 in finch brains was very similar, as expected, though not quite identical. ^{125}I-α-bungarotoxin showed an overlapping but distinct pattern of binding, as expected.[66,67]

Nicotinic receptors were localized by indirect immunofluorescence and indirect immunoperoxidase labeling of chicken retinas with mAbs 210 and 270.[68] This is a collaborative study with Drs. Kent Keyser and Harvey Karten at the University of California, San Diego. Formalin-fixed tissue was used to permit cell-level resolution, but fixation may alter receptor antigenicity. mAb 270 recognizes the structural subunits of both receptor subtypes in brain and may bind to ganglionic receptors.[20] mAb 210 to the MIR of muscle receptors binds strongly to the brain receptor subtype with 59,000 molecular weight acetylcholine-binding subunits, but not to the α4 subtype.[20] On Western blots it binds to structural subunits common to both

subtypes, but its epitope may be obscured in native α4-type receptors. Like mAb 35, mAb 210 binds well to the α3-type ganglionic receptors in chicken ciliary ganglia. Figure 14 shows that retina contains a great deal of structural subunit and many more α3- than α4-type acetylcholine-binding subunits. mAb 210 and mAb 270 gave similar labeling patterns in retina, but mAb 210 was more effective. Figures 16-18 illustrate labeling of retinas with mAb 210.

mAbs 210 and 270 labeled cells in the inner nuclear layer and ganglion cell layer of chicken retinas, including ganglion cells, displaced ganglion cells, and amacrine cells. Approximately 12-18% of ganglion cells were labeled. The retina provides a small, relatively accessible and well-characterized piece of central nervous tissue in which we can begin to work out the techniques and data necessary to understand the functional roles of nicotinic receptor subtypes. This will ultimately involve differentially localizing receptor subtypes at high resolution, localizing both the proteins and the mRNAs which code them (as in Figure 19), and localizing the receptors with respect to other cells, such as those which release acetylcholine (as in Figure 18).

Nicotinic receptors were immunolocalized in frog brains at both the light and electron microscopic level in collaborative experiments with Dr. Peter Sargent of the University of California, Riverside.[69] Previously, our library of mAbs to receptors from electric organs were tested for ability to bind to receptors in frog muscle.[54] A subset of 28 of these mAbs was recently found to bind to the optic tectum of Rana pipiens.[69] Most of these were directed against the MIR. Of these, mAb 22 was used most extensively. This mAb labeled a subset of retinotectal projections (Figure 20). Electron microscopy revealed that labeling was associated with extrasynaptic regions (Figure 21). As in goldfish,[70,71] chickens,[67] and rats,[67] removal of one retina resulted in the loss of immunoreactivity in the contralateral tectum (Figure 22). Labeling was found in the optic tract and associated with retinal ganglion cells. Thus, in all species examined, nicotinic receptors on central processes of retinal ganglion cells are a prominent feature.

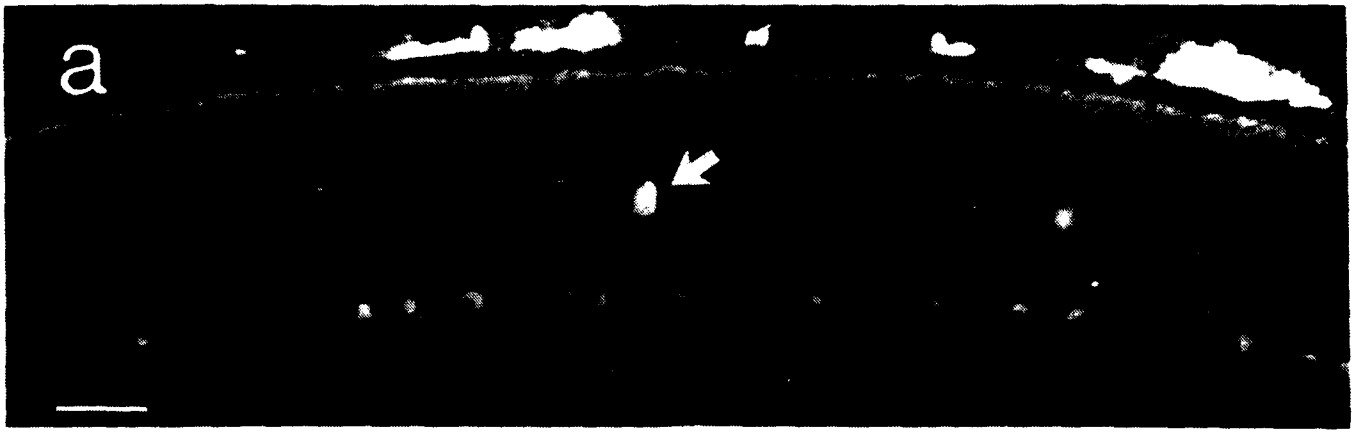
Structure	*Relative staining intensity* α-*bungarotoxin*	*mAb 35*	*mAb 270*
nucleus accumbens	1	1	1
nucleus basalis	1	1	1
nucleus cerebelli intermedium	2	3	3
nucleus cerebelli medialis	2	3	3
nucleus cuneatus externus	1	1	2
ectostriatum	1	1	1
fasciculus prosencephali lateralis	3	1	2
substantia grisea centralis	2	2	2
nucleus geniculatus lateralis, pars ventralis	2	4	4
hyperstriatum accessorium	4	1	1
hyperstriatum dorsalis	3	3	3
nucleus habenula medialis	2	3	3
hippocampus	3	3	3
hyperstriatum ventralis	2	3	3
nucleus hyperstriatum ventralis, pars caudalis	3	1	1
nucleus intercollicularis	2	1	3
nucleus isthmi, pars magnocellularis	1	3	3
nucleus isthmi, pars parvocellularis	2	2	2
nucleus laminaris	1	3	1
nucleus lentiformis mesencephali	3	3	3
locus ceruleus	3	3	3
lobus parolfactorius	1	1	1
nucleus magnocellularis, anterior neostriatum	3	1	1
nucleus mesencephalicus lateralis, pars dorsalis			
(peripheral shell)	3	3	3
(core region)	1	1	1
nucleus motorius nervi trigemini	3	3	3
neostriatum	2	1	1
nucleus nervi trochlearis	3	3	3
nucleus nervi abducens	3	3	3
nucleus nervi facialis	3	3	3
nucleus motorius dorsalis nervi vagi	3	3	3
nucleus nervi hypoglossi	3	1	1
nucleus olivaris inferior	4	1	2
nucleus nervi occulomotorii	3	3	3
tractus occipitomesencephalicus	1	1	1
paleostriatum augmentatum	2	1	2
nucleus pontis lateralis	1	3	1
nucleus pontis medialis	1	3	1
nucleus medialis hypothalami posterioris	3	1	3
nucleus preopticus medialis	3	1	3

Structure	*Relative staining intensity*		
	α-bungarotoxin	*mAb 35*	*mAb 270*
nucleus pretectalis	3	3	3
periventricular organ	4	1	1
nucleus robustus archistriatalis	1	1	1
nucleus reticularis gigantocellularis	1	3	3
nucleus reticularis lateralis	1	3	3
nucleus reticularis pontis caudalis	1	1	3
nucleus reticularis parvocellularis	1	1	2
nucleus rotundus	1	1	1
nucleus tractus solitarii	1	3	3
nucleus semilunaris	3	1	2
nucleus septalis medialis	2	1	2
nucleus subpretectalis	1	3	3
nucleus superficialis parvocellularis	1	2	2
nucleus spiriformis lateralis	1	4	4
torus semcircularis	2	1	2
tectum opticum:			
striatum opticum	3	2	2
stratum griseum & fibrosum superficialis	3	3	3
stratum griseum centrale	2	3	3
stratum album centralis	1	1	1
substantia grisea et fibrosa periventricularis	2	1	2
tractus opticus	1	1	1
nucleus et tractus descendens nervi trigemini	1	3	3
nucleus vestibularis dorsolateralis	2	3	2
nucleus vestibularis lateralis	2	3	2
nucleus vestibularis medialis	3	2	3

Figure 15. Summary of the pattern of nicotinic ligand labeling in the Zebra finch brain. *Reproduced from reference 65.*


a
ML
IPL
GCL
b
c

Figure 16. Photomicrographs and a drawing of cross sections of mAb 210-labeled retinas. *The top panel (a) illustrates the distribution of immunoreactivity in a 10-μm-thick transverse section labeled with a fluorescein-conjugated secondary antiserum. Labeled somata visible in both the inner nuclear layer (INL) and ganglion cell layer (GCL) gave rise to processes that arborized in two distinct laminae of the inner plexiform layer (IPL). The middle panel (b) illustrates similar features in an avidin-biotin-horseradish-peroxidase-reacted, 20-μm-thick section. Note, however, the large cell visible in the INL. The lower panel (c) is a camera lucida drawing of a section processed as in panel (b). This illustrates that dendrites could be followed from the labeled somata (stippled) in the INL and GCL into two laminae of the IPL. The processes often extended beyond the more proximal band of labeling and terminated in the more distal one. Scale bar = 50 μm in (a); 50 μm in (b); 30 μm in (c). Reproduced from reference 68.*


a

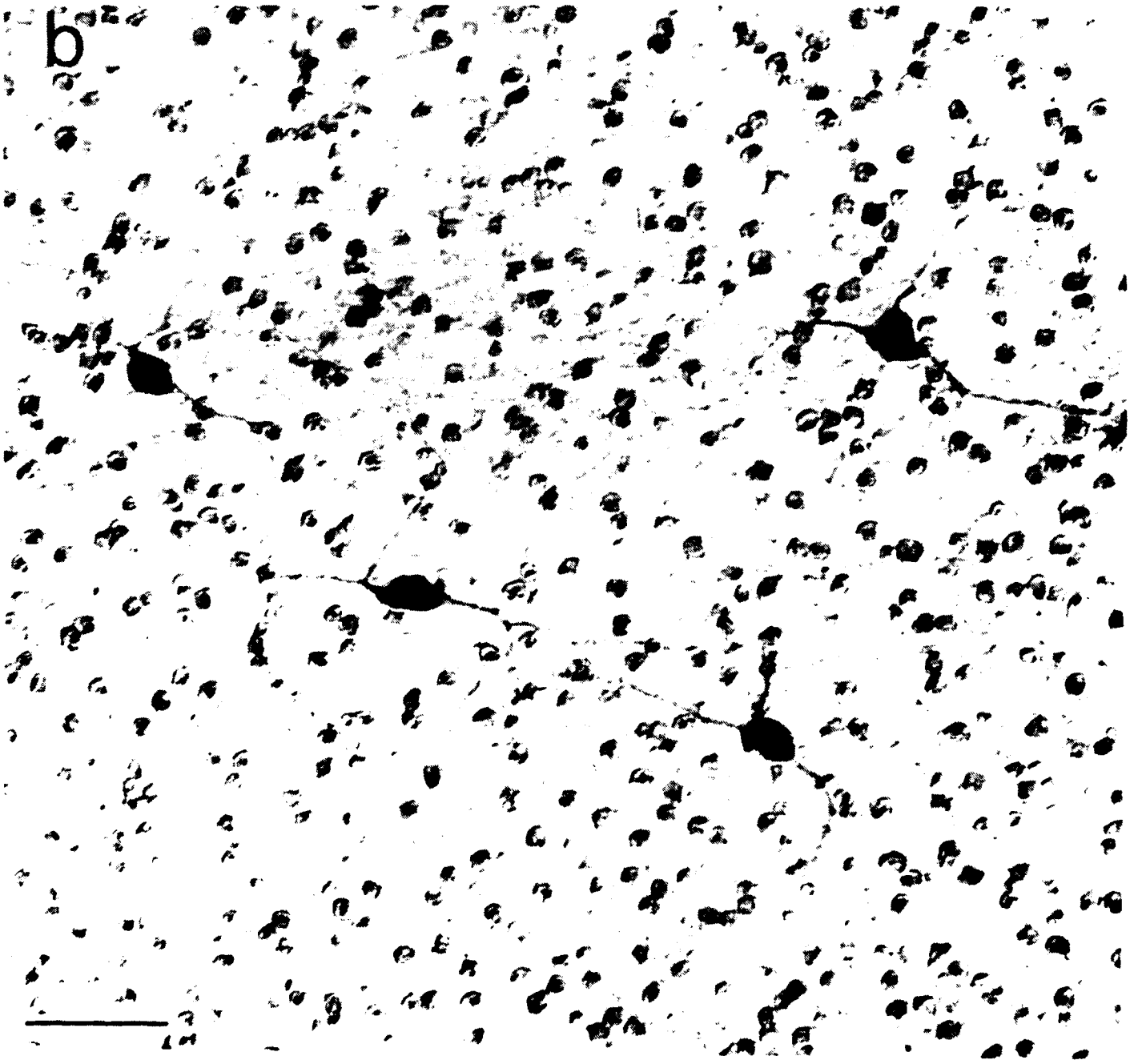

b

Figure 17. Photomicrographs illustrating a class of large mAb 210-labeled cells in the INL. In (a), a cross section, a large labeled soma is visible in the inner INL (arrow) and its processes enter the outermost band of immunoreactivity in the IPL. The lower panel (b) illustrates four of these cells in a 30-μm-thick horizontal section. Note the extensive, overlapping dendritic fields. Scale bar = 50 μm in (a); 50 μm in (b). Reproduced from reference 68.

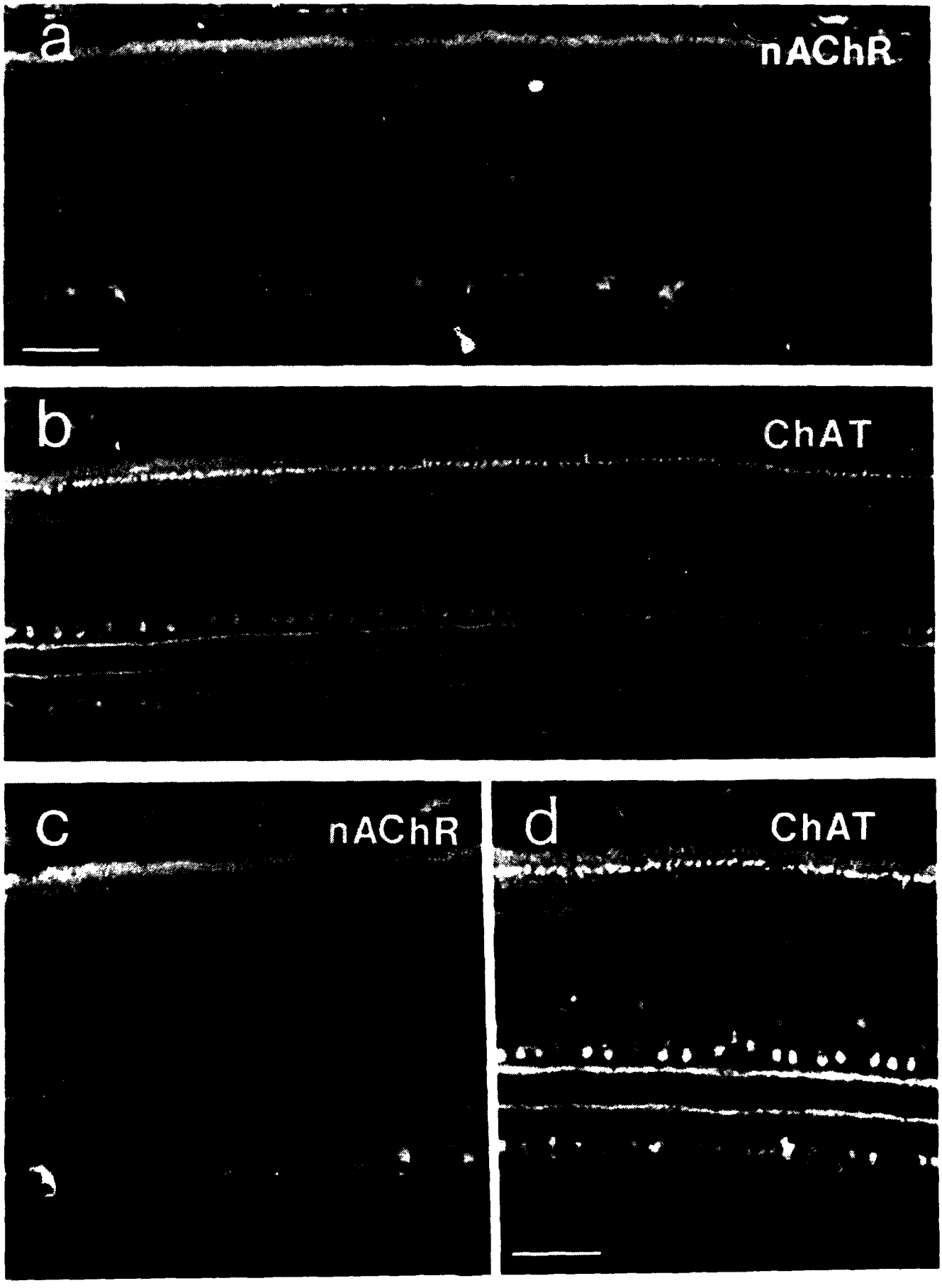

a
nAChR
b
ChAT
c
nAChR
d
ChAT

Figure 18. Fluorescence micrographs of a transverse section of retina illustrating the distribution of mAb 210-positive cells and processes and the corresponding pattern of choline-acetyltransferase (ChAT) immunoreactivity in the same section. These panels illustrate that the patterns of immunoreactivity in the INL, IPL, and GCL were similar (a,b) but that the ChAT-positive cells were smaller and more numerous than receptor-positive cells. Panels (c,d) are contiguous portions of the same section placed side by side to illustrate that the arborization patterns of the two cell types were in register. However, the band of mAb 210-positive immunoreactivity in lamina 2 extended inward more than did the corresponding ChAT-positive band. Scale bar = 50 μm for (a) and (b); 50 μm for (c) and (d). Reproduced from reference 68.

A. in situ hybridization B. monoclonal antibody

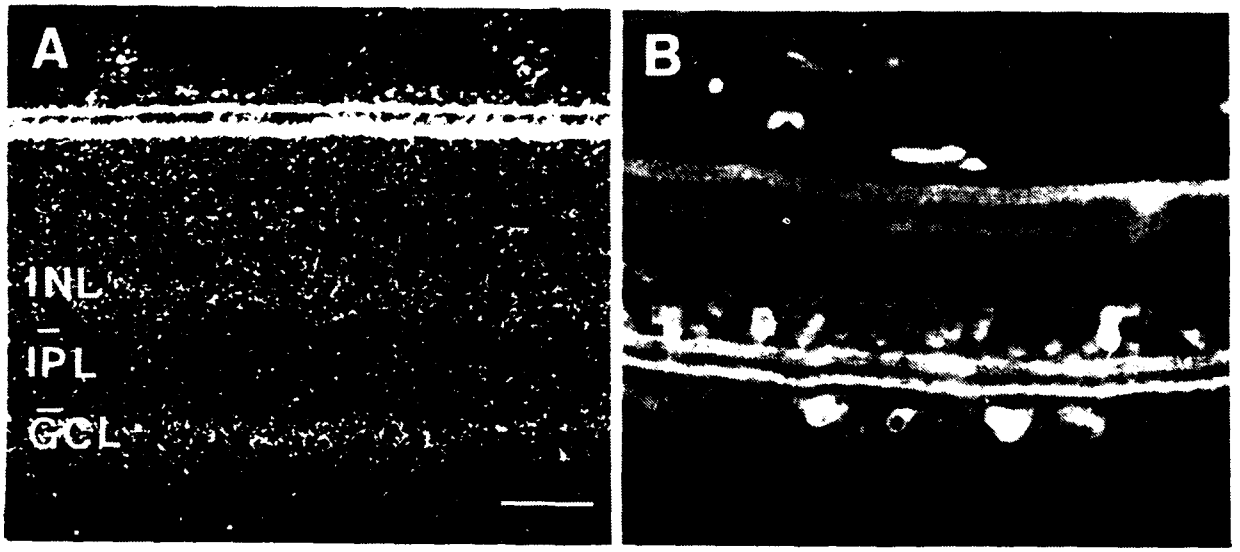


Figure 19. Localization of the structural subunit in chicken retina. *(A) mRNA for the structural subunit is localized using an ^{35}S-labeled anti-sense RNA probe corresponding to the putative cytoplasmic loop (amino acids 306 to 410) of the structural subunit. Cell bodies in the GCL are labeled, as are cells in the INL, whereas dendritic processes in the IPL are not heavily labeled. The pigment layer produces an intense artifactual birefringent double layer at the top of this darkfield micrograph. A control sense cRNA probe gave uniform low background labeling. The scale bar is 40 μ. (B) Structural subunit protein is localized by indirect immunofluorescence using mAb 270. The subunit protein is localized in the cell bodies of ganglion cells and displaced ganglion cells, as well as in the dendritic and axonal processes of these cells, and some others, located in the inner and outer plexiform layers. The pigment layer at the top appears dark in this fluorescence micrograph. The sections in A and B are from different parts of the retina and differ in sizes. Reproduced from reference 11.*

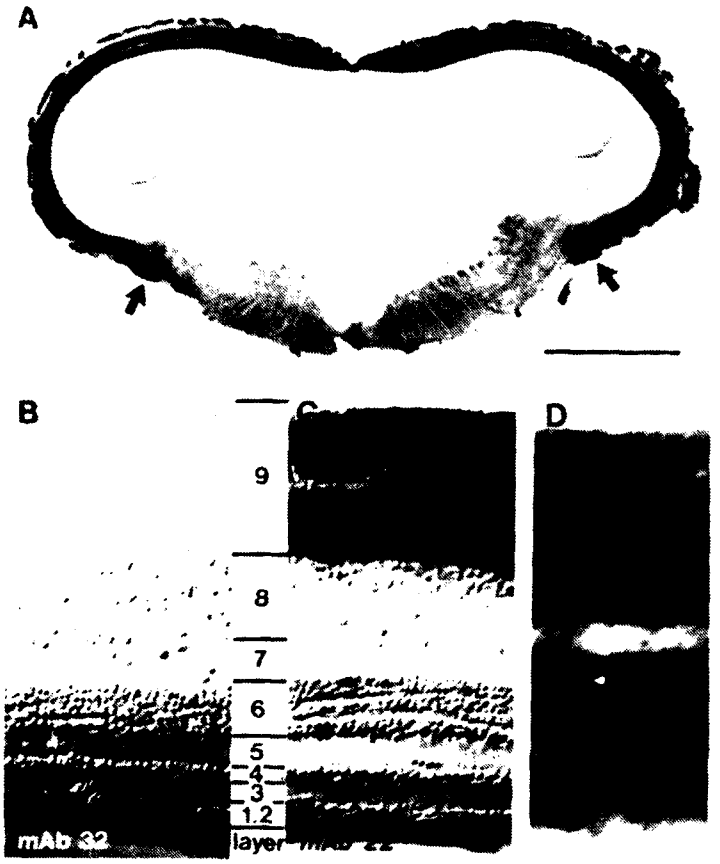


Figure 20. Immunoperoxidase staining of the optic tectum using anti-receptor mAb 22. *(A) Low-power, bright-field photomicrograph of a 100 μm Vibratome section from the midbrain of Rana pipiens. Peroxidase staining is found within the superficial parts of the tectum and extends to the lateral optic tracts (arrows). Density at the base of the tectum (between the 2 arrows) represents cobalt staining of myelin tracts and not horseradish peroxidase reaction product. (B) and (C) Higher-power differential interference contrast (Nomarski) photomicrographs after incubating Vibratome sections with the control mAb 32 (B) and the cross-reactive mAb 22 (C) and visualizing mAb binding using the avidin biotinylated-horseradish peroxidase technique. Strong staining is observed after using mAb 22, but not mAb 32 (density at bottom of slices is shadowing due to Nomarski optics). The vertically oriented set of numbers between (B) and (C) refer to the layers of the tectum, extending from the ventricular surface (layer 1) to the pia (layer 9). (D) Banding pattern of stain within the neuropil at high magnification (bright-field optics). The entire field in (D) corresponds to layer 9. Distinct bands of stain are visible. Scale bar (in A): 1 mm in (A), 150 μm in (B,C) and 60 μm in (D). Reproduced from reference 69.*

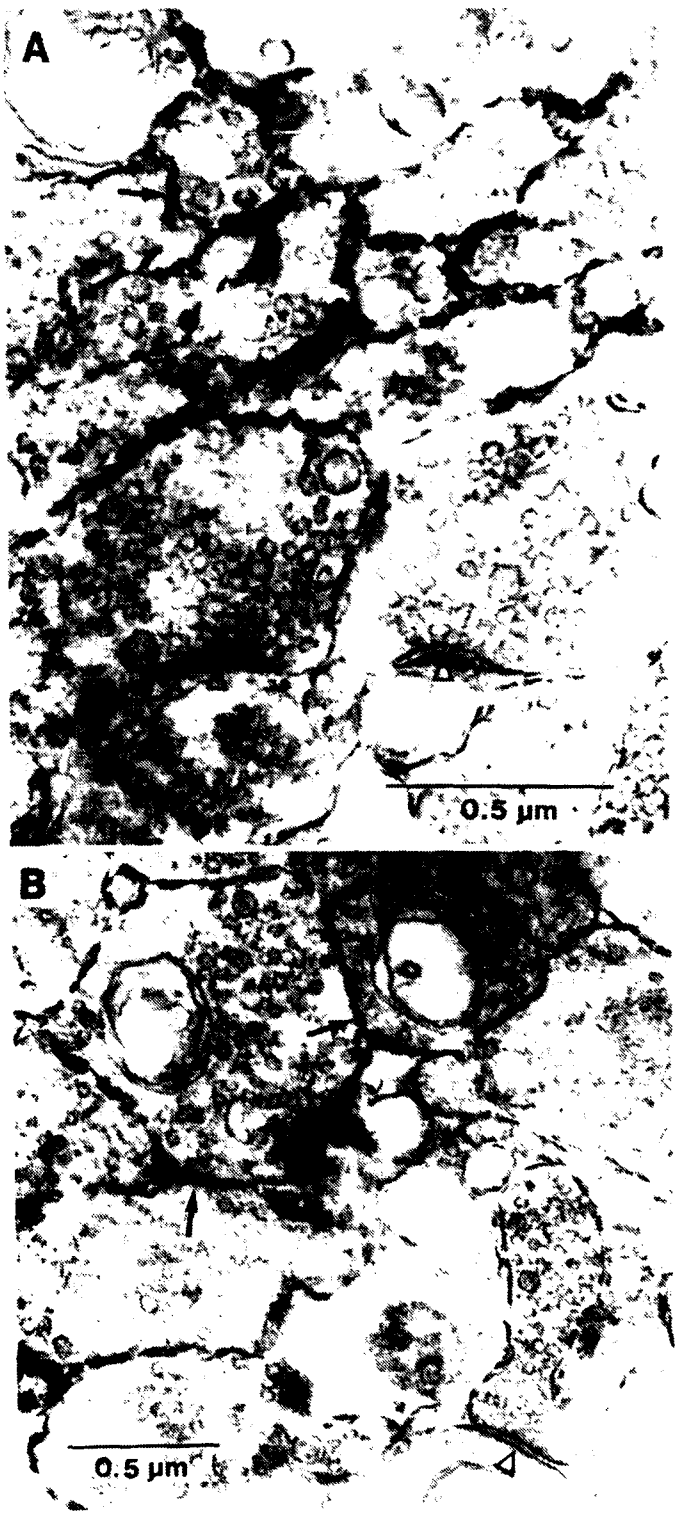


Figure 21. Electron micrographs of the optic neuropil showing extrasynaptic location of receptor-like immunoreactivity. (A) and (B) show two fields taken from the neuropil. Peroxidase stain obtained using mAb 22 and the avidin-biotin technique (arrows) is associated with membranes but not with either the pre- or the postsynaptic membrane at synapses (open arrowheads). Reproduced from reference 69.

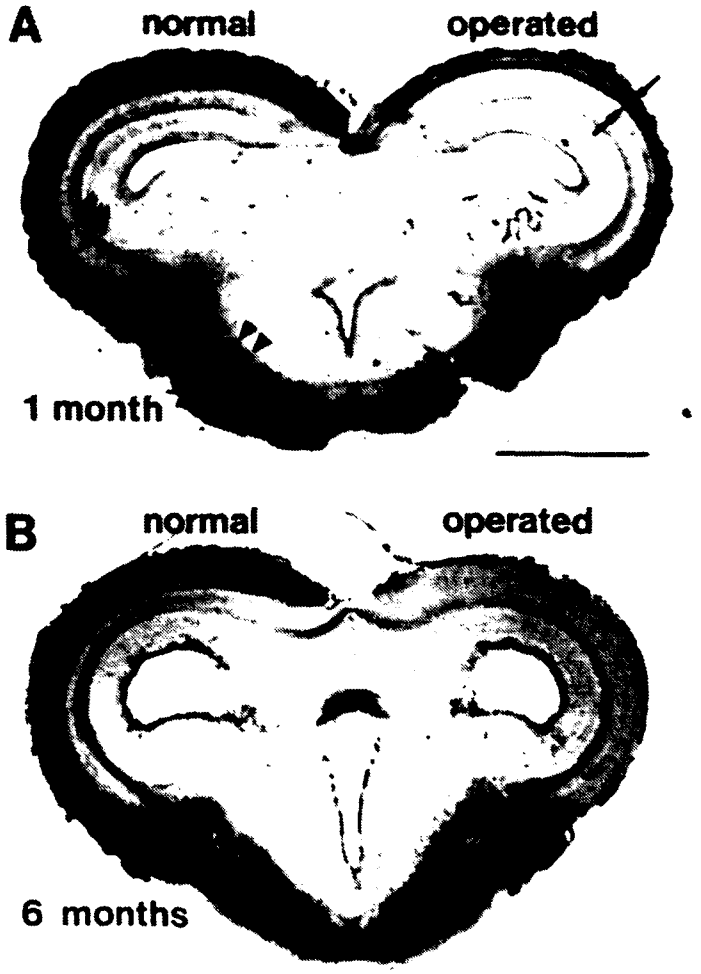


Figure 22. Loss of immunoreactivity following removal of the retina. *(A) Removal of one retina results in partial loss of mAb 22 binding in the contralateral tectum 30 days after surgery. The residual staining is located in bands corresponding to projection layers "a," "c," and "e." Staining associated with layer "c" (delineated by the arrows) is most obvious, while that associated with layers "a" and "e" are most evident near the lateral optic tract. Matsumoto and Scalia[93] showed that projection layers "a," "c," and "e," which are unmyelinated, survive for long periods after eye enucleation. (B) Removal of the retina 6 months prior to staining resulted in a complete loss of immunoreactivity. Staining at the base of the tectum on both normal and operated sides (see arrowheads in A) corresponds to cobalt staining of myelinated tracts and not to horseradish peroxidase reaction product. Scale bar (in A): 1 mm. Reproduced from reference 69.*

Neuronal α-Bungarotoxin-Binding Protein

Previously, we had observed a pattern of four bands resembling the subunits of muscle nicotinic receptors in α-bungarotoxin-binding proteins affinity purified from rat brains.[22] However, without a library of subunit-specific mAbs, it could not immediately be determined which of these bands corresponded to subunits and which, if any, corresponded to contaminants, nor could it be determined whether this pattern arose from the superposition of subunits of several subtypes of this protein (as had been the case with two subtypes of nicotinic receptor immunoaffinity purified using a mAb specific for a subunit common to both subtypes[22]).

We prepared a high-titer rat antiserum to α-bungarotoxin-binding proteins affinity purified from chicken brains.[19] So far, one rat mAb to this protein has been obtained. At this time several mouse mAbs to this protein are in the process of recloning. We will use these mAbs to characterize the subunit structure of these proteins as we did the structure of neuronal nicotinic receptors.

Conti-Tronconi et al.[39] reported a partial N-terminal amino acid sequence for one of the subunits of α-bungarotoxin-binding proteins from chicken brains. This sequence suggested homology with nicotinic receptors.

Using an oligonucleotide based on this partial sequence, we screened our chicken brain cDNA library for homologues. This identified a partial-length cDNA nearly identical to the probe sequence. This cDNA clearly coded for a previously unidentified nicotinic receptor subunit homologue. By screening with this cDNA, a distinct but related full-length homologue was identified which has a cysteine pair that may be associated with an acetylcholine binding site. Other homologues are being sought.

To test the identity of these cDNAs, unique peptide segments are being expressed in bacteria. These will be used as antigens to see if they react with the antisera and mAbs we have to α-bungarotoxin-binding proteins, and they will be used as immunogens to raise antisera to test on native α-bungarotoxin-binding proteins. Other tests will follow.

For the first time we may be beginning to get a grip on the molecular identity of the α-bungarotoxin-binding protein. This may ultimately help us to understand the function of this enigmatic protein.

Sucrose Gradient Sedimentation Analysis of Receptors from TE671 Cells

TE671 cells were grown in Iscove's modified Dulbecco's medium with 5% bovine calf serum, as previously described.[19] The cells were harvested and receptors extracted in 1% Triton X-100 detergent, 50 mM Tris, pH 7.5, 150 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 5 mM IAA, 5 mM aminobenzamidine, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml bestatin, 10 μg/ml Trasylol, and 10 μg/ml soybean trypsin inhibitor, as also previously described. Aliquots of the extract (150 μl) were layered onto 5 ml sucrose gradients (5-20% sucrose wt/wt, in 0.5% Triton X-100, 10 mM Na phosphate buffer, pH 7.5, 100 mM NaCl, 1 mM NaN_3). The gradients were centrifuged in a VTi 65.2 rotor (Beckman, Fullerton, CA) for 67 minutes at 4°C. The gradients were fractionated from the bottom of the tubes and collected into Immulon "C" Removawells (Dynatech, Chantilly, VA) coated with mAb 210 (1 μg/well, 4 hours, followed by 3 washes). After incubating overnight at 4°C with shaking, the wells were washed three times with 0.5% Triton X-100 in 100 mM NaCl, 1 mM NaN_3, 10 mM Na phosphate buffer, pH 7.5. ^{125}I-α-bungarotoxin was then added at 2 nM in the Triton X-100 buffer, and 100 mM carbamylcholine was added as a competitive inhibitor on some gradients to address affinity for small cholinergic ligands, while unlabeled α-bungarotoxin was added at 1 μM to other gradients to determine the level of nonspecific binding. After overnight incubation the wells were washed three times, and then bound ^{125}I-α-bungarotoxin was determined by γ counting. Receptor from Torpedo electric organ was analyzed simultaneously to provide size markers.

Expression of Torpedo Nicotinic Receptor Subunits

E. coli HB 101 was used to amplify plasmid DNA.[72] E. coli DH5α was used for sequencing purposes. Saccharomyces cerevisiae strain TD4 (Mat a, his4, leu2, ura3, trp1) and strain 8534-8C (Mat α, his4, leu2, ura3) were from B. Tye (Cornell University), and strain TD71.8 (Mat α, his3, leu2, ura3, trp1, lys2) from D. Dawson (Massachusetts General Hospital). The negative control strain for the production of subunit polypeptides in yeast, KUJ6.0, was obtained by mating TD4 with TD71.8.

The full-length β subunit cDNA was isolated as an Eco RI restriction fragment from clone pSS2β.[73] The γ subunit cDNA was obtained as an NcoI-PvuII restriction fragment from clone γ-28.[74] The ends were made flush with Klenow fragment, and Eco RI linkers (#5295LC, Bethesda Research Laboratories, Bethesda, MD) were added according to standard procedures.[72] The Eco RI restriction fragments were inserted individually into the yeast expression vector pMAC561[75] to generate pYTcβ or pYTcγ. Together with pYTcα and pYTcδ, which were obtained previously,[44-46] the vectors were used for the construction of the integrating expression vectors.

Plasmids pD41 and pSZ62 containing the yeast selectable markers TRP1 and HIS3, respectively, were obtained from D. Dawson (Massachusetts General Hospital), and pSZ58 containing the LEU2 gene of yeast was from B. Tye (Cornell University). Yeast transformation was performed using the lithium acetate method.[76] For E. coli transformation the $CaCl_2$ procedure was used.[72] Sequence analysis was done according to the manual supplied with the Sequenase sequencing kit (United States Biochemicals, Cleveland, OH). All other DNA manipulations were performed according to standard procedures.[72]

SP6 mRNA transcripts from Torpedo receptor subunit cDNAs were made in vitro as described.[77] Yeast total RNA was prepared, with minor variations, according to Silverman et al.[78] Poly $(A)^+$ RNA was purified from this total RNA by chromatography on oligo (dT)-cellulose.[79]

For the detection of the receptor α, γ, and δ subunits, yeast strains were grown at 30°C or at 5°C in SD medium[80] (Difco, Detroit, MI). For the detection of the β subunit, 5 ml of a preculture were grown in SD medium at 30°C to an optical density measured at 600 nm of >0.6, then diluted into 500 ml fresh medium and grown for an additional 5 hours at 30°C; the cells were then incubated for 5 days at 5°C until the optical density was 0.3. Cells were harvested from 500 ml cultures by centrifugation at 100g for 20 minutes at 5°C. All further treatments of the cells to obtain the membrane fraction were also carried out at 5°C. The cell pellet was washed twice in 500 ml of 50 mM Tris-HCl, 100 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 25 mM NEM, pH 7.5. After freezing the pellet at -80°C for at least 30 minutes, it was suspended in 20 ml of 25 mM Tricine-KOH, 0.6 M sorbitol, 50 mM KF, 1 mM PMSF, 25 mM NEM, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml soybean trypsin inhibitor, pH 7.5. The suspension was sonicated for 5 minutes and then centrifuged at 1100g for 5 minutes. The supernatant was saved and the pellet treated as before in 20 ml of the above buffer. The last supernatant was combined with the previous one and centrifuged at 3000g for 10 minutes. The resulting supernatant was then centrifuged at 300,000g for 30 minutes, to obtain the yeast membrane fraction.

For immunoblot analysis yeast membrane proteins were solubilized in SDS-sample buffer,[81] and proteins were separated by SDS-polyacrylamide gel electrophoresis using a 10% gel. Proteins were then transferred to Immobilon membranes (Millipore, Bedford, MA) by the method of Matsudaira.[82] The membranes were quenched with 5% (w/v) dried milk in phosphate-buffered saline, 0.5% Triton X-100, pH 7.5, and then labeled with iodinated mAbs against the individual subunits (specific radioactivities 1-2 x 10^8 cpm/mol). The mAbs were iodinated using a modified chloramine-T procedure.[83] The membranes were washed with phosphate-buffered saline, 0.5% Triton, pH 7.5, and bound ^{125}I-mAbs were visualized by autoradiography.

For solid-phase sandwich assay of mAb and α-bungarotoxin binding to Torpedo nicotinic receptor α subunits expressed in yeast, Immobilon "C" microtiter wells (Dynatech) were coated with 1 μg of mAb 173 in bicarbonate buffer (10 mM $NaHCO_3$, pH 8.8, 50 μl/well) for 4 hours at room temperature. After three washes the wells were quenched for a further 2 hours with 1% bovine serum albumin in the same buffer. Finally the wells were washed three times with 200 μl of 0.5% Triton X-100 in phosphate-buffered saline. Aliquots (50 μl) of crude extracts of yeast membranes, purified receptor, or α subunits in 0.5% Triton X-100 buffer were incubated in the wells overnight at 4°C with shaking. After three washes the ^{125}I-labeled ligand ($3 \times 10^{17} - 3 \times 10^{18}$ cpm/mol) was added in 50 μl of 1% bovine serum albumin, 1% ovalbumin, 0.1% Tween 20, and 10 mM Na phosphate, pH 7.5. After shaking overnight at 4°C, the wells were washed three times and counted in a γ counter.

Mapping the Binding Site of mAbs to the MIR Using Synthetic Peptides

Some of the synthetic peptides were reported previously,[51] whereas the Torpedo and human α subunit peptides around α66-76 were synthesized using the RaMPS multiple peptide synthesis system (DuPont, Wilmington, DE) according to the manufacturer's directions.

Microtiter plates (Immulon, from Dynatech) were coated with poly-D-lysine HBr (20 μg/ml, 100 μl/well) in 0.1 M $NaHCO_3$ at 4°C overnight. All subsequent treatments were at room temperature. The plates were washed three times with 10 mM K phosphate buffer, pH 7.0. Peptides at 5 μM in this buffer were mixed with an equal volume of 0.25% glutaraldehyde and 10 μl aliquots were added to the wells. After 2 hours the wells were washed three times with buffer and then quenched with 200 μl of 1% bovine serum albumin, 1% ovalbumin, 0.1% Tween 20 in 10 mM Na phosphate, pH 7.5. After shaking for 1 hour, this solution was removed, and mAbs were added at 50 μl/well containing about 1×10^{-10} mol of mAb in the same buffer. After shaking for a further 2 hours, the wells were washed three times with 200 μl of 0.05% Tween 20 in phosphate-buffered saline. A mouse anti-rat IgG mAb (MAR 18.5) conjugated to horseradish peroxidase was then added at 50 μl/well. After shaking for 1 hour, the wells were washed three times with the 0.05% Tween 20 buffer. Then 150 μl/well of peroxidase substrate solution (20 mg 2,2'-azino-di-[3 ethyl-benzthiazolinsulfonate] in 72 ml phosphate-citrate buffer, pH 4.0, with 22 μl of H_2O_2) was added. After color development for 20 minutes the plate was read in a Titertek Multiskan MCC/340 (Flow Laboratories, McLean, VA).

Subunit Stoichiometric Determination of Neuronal Nicotinic Receptors

Torpedo nicotinic receptors were purified as previously[83] described, with some modifications. Briefly, a high capacity (10 mg toxin/ml Sepharose) Naja naja toxin Sepharose column was

used to bind the receptor, which had been solubilized in Thesit (Boehringer Mannheim), rather than Triton X-100. Receptor bound to the affinity column was then eluted by recirculation of 1 M carbachol in 25 mM Tris pH 7.5, 1.0% Thesit, through a hydroxylapatite (BioRad, Richmond, CA) column, from which it was subsequently eluted with 50 ml of 150 mM NaP pH 7.5, 1.0% Thesit.

Chicken brain nicotinic receptors were purified as described previously, with some modifications.[20] Membranes were prepared from 150 g of chicken brains and then extracted in 2 volumes of 2% Triton X-100 buffer. The detergent extract was subsequently shaken overnight with 3 ml of mAb 289 (directed to the 75 kD ACh-binding subunit) coupled to AFC resin (New Brunswick Scientific, Edison, NJ) at 8 mg/ml. The column was washed first with 100 ml of 10 mM Na phosphate buffer pH 7.5, containing 1 M NaCl, 1 mM EDTA, 1 mM EGTA, and 0.5% Triton X-100; and then washed with 25 ml of 10 mM Na phosphate buffer pH 7.5, containing 100 mM NaCl and 0.1% Thesit. Bound receptor was then eluted with 7 ml of 50 mM Na citrate buffer pH 3.0, containing 0.05% Thesit. After neutralization the eluate was concentrated using a Centricon 30 (Amicon, Danvers, MA) and lyophilized.

Rat brain receptors were purified from 130 g of rat brains using mAb 295-AFC, as previously described for bovine brain receptors,[23] with the modification that before elution of the bound receptor, the affinity column was washed with 25 ml of 10 mM Na phosphate buffer pH 7.5, containing 100 mM NaCl and 0.1% Thesit. Receptors were then eluted with 5 ml of 50 mM Na citrate buffer pH 3.0, containing 0.05% Thesit. The eluate was neutralized, concentrated and lyophilized as above.

Bovine brain receptors were purified as described previously,[23] concentrated, and lyophilized as described above.

Purified receptors were labeled with ^{125}I as follows. To 20-100 pmol of receptor, in 25 μl of 10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl, 1% SDS, and 4 M urea, was added (i) 50 μl of 0.4 M Na phosphate buffer, pH 7.5, containing 2% SDS and 8 M urea, (ii) 50 μl Enzymobeads (BioRad), (iii) 2μl $Na^{125}I$ (1 mCi), (iv) 25 μl 10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl, 1% SDS, 4 M urea, and 1% β(D) glucose. The reaction was allowed to proceed for 90 minutes at room temperature before 20 μl of 1M NaN_3 were added to stop the reaction. The radioiodinated protein was separated from free ^{125}I using a BioRad Econo-Pac 10 DG disposable desalting column. The column was pretreated with 20 mg of β-lactoglobulin (Sigma) to reduce nonspecific binding, and then equilibrated in buffer (10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl, 1% SDS, 4 M urea, 10 mg/ml β-lactoglobulin).

Approximately 0.5-10.0 x 10^5 cpm of ^{125}I-labeled receptor were loaded onto single lanes of 10% SDS polyacrylamide gels and resolved by electrophoresis (in SDS). The gels were fixed, dried, and autoradiographed. The dried gels were aligned with

the autoradiograms, the pieces of the gels corresponding to the subunit bands excised, and radioactivity quantitated by γ counting. For ^{125}I rat brain receptor the excised subunits contained 2-5 x 10^5 cpm; ^{125}I bovine brain receptor subunits contained 3.5-4 x 10^5 cpm; ^{125}I Torpedo receptor contained 3-7 x 10^5 cpm; and ^{125}I chicken brain receptor contained about 3 x 10^3 cpm per excised band. The radioactivity per subunit was then normalized for the number of tyrosine residues present in each subunit, as determined from the deduced amino acid sequences: In Torpedo receptor α, β, γ, and δ subunits[16] there are 17, 16, 17, and 19 tyrosine residues, respectively; in the chicken brain receptor structural subunit[25] there are 18 tyrosines, and in the chicken brain acetylcholine-binding subunit[26,32] there are 21 tyrosines; in the rat brain receptor structural subunit[31] there are 15 tyrosines, and in the acetylcholine-binding subunit[29] there are 17. No cDNA sequences are available for bovine brain receptor subunits. However, since they are certainly very homologous to the chicken and rat brain receptor subunit sequences, which have a ratio (acetylcholine-binding subunit : structural subunit) in the number of tyrosines of 1.0 : 0.86 (chicken) and 1 : 0.88 (rat), the normalization value of 0.87 was used for bovine receptors. After normalization of the number of tyrosine residues, the ratio of cpm was determined between subunits within a single lane to eliminate pipetting errors. The means of the ratios of 6-11 lanes were then determined.

Sucrose gradient analysis of neuronal nicotinic receptors was conducted as follows. Chicken and rat brain receptors were extracted from crude membranes for 2 hours, using an equal volume of 2% Triton X-100 in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM p-aminobenzamidine, 5 mM iodoacetamide, and 0.5 mM PMSF. Day 18 embryonic chicken brains were used in these experiments because they have a higher concentration of receptors than adult brain (unpublished observation). Torpedo receptors, which were used as an internal control for each gradient, were similarly extracted with Triton X-100 from crude membranes of Torpedo electric organ. The Torpedo receptors (77 nM) were trace-labeled with ^{125}I-α-bungarotoxin (10 nM) and then diluted into the brain extracts to give ~40,000 cpm ^{125}I-α-bungarotoxin-labeled Torpedo receptor per gradient. Aliquots (450 μl) of the brain extracts containing the ^{125}I-α-bungarotoxin-labeled Torpedo receptors were layered onto 11 ml sucrose gradients (5-20% sucrose wt/wt, in 10 mM Na phosphate buffer, pH 7.5, containing 100 mM NaCl, 1 mM NaN_3, and 0.5% Triton X-100). The gradients were centrifuged for 17 hours at 41,000 rpm in a Beckman SW41 Ti rotor. Thirty drop fractions were subsequently collected from the bottom of the tubes and analyzed by γ counting to determine the sedimentation of the Torpedo receptors. The brain receptors in the gradient fractions were quantitated by [^{3}H]-L-nicotine binding using a mAb-immobilization assay. Gradient fractions were shaken overnight at 4°C with 25 μl of a 1:1 slurry of goat anti-rat immunoglobulin-Sepharose and 1 μl of a stock solution of mAb 270. The Sepharose was washed with 2 x 1 ml of 10 mM Na phosphate

buffer, pH 7.5, containing 100 mM NaCl and 0.5% Triton X-100 (PBS-Triton), and incubated with 20 nM [^{3}H]-L-nicotine for 15 minutes at room temperature. The Sepharose was then washed with 3 x 1 ml of PBS-Triton by repeated centrifugation and resuspension. A solution of 2.5% SDS and 5% 2-mercaptoethanol (100 μl) was added to the Sepharose pellet, the entire contents were then added to a 5 ml T2 5% scintillation cocktail, and the radioactivity was determined by liquid scintillation counting. Control tubes containing only buffer solutions were included in each assay to determine nonspecific [^{3}H]-L-nicotine binding. To determine the extent of contamination of each tube by residual ^{125}I-α-bungarotoxin, the tubes were again analyzed by γ counting after the [^{3}H]-L-nicotine binding assay. In no cases did the gradient fractions contain >100 cpm ^{125}I when analyzed at this time, thus indicating minimal contamination by ^{125}I when counting ^{3}H.

Rat brain receptors were also extracted from crude membranes for 2 hours, using an equal volume of 2% cholate and 0.2% asolectin in the same buffers used for extraction in Triton X-100. Likewise, Torpedo receptors were extracted from crude membranes of electric organ with the same detergent-lipid mixture. The Torpedo receptors were again prelabeled with ^{125}I-α-bungarotoxin and diluted into the rat brain extract. Aliquots (450 μl) of this extract were layered onto 11 ml sucrose gradients (5-20% sucrose wt/wt, 10 mM Na phosphate, pH 7.5, 100 mM NaCl, 1 mM NaN_3, 2% cholate, and 0.2% asolectin). The gradients were centrifuged for 17 hours in a SW41 Ti rotor at 41,000 rpm and fractionated by collecting 20 drops/fraction from the bottom of the tubes. The Torpedo and brain receptors in each fraction were quantitated as described above.

Neuronal Nicotinic Receptor Subunit Characterization, cDNA Cloning, Bacterial Expression, and Expression During Chicken Development

The N-terminal amino acid sequences of receptor subunits were determined as described previously.[24] Receptors from chicken brain were purified by affinity chromatography upon mAb 270 and the receptor subtype with a 75,000 molecular weight acetylcholine-binding subunit isolated by a second round of affinity chromatography using mAb 299.[20] Purified receptor (~60 pmol) was resolved into subunits by electrophoresis in a 10% polyacrylamide gel in SDS, then electroblotted onto a quaternary ammonium-derivatized glass fiber sheet. Protein bands were located by fluorescent staining, excised and subjected to gas phase microsequencing[84] using an Applied Biosystems model 470A protein sequencer (Foster City, CA).

A cDNA library was prepared in the λ Zap cloning vector (Stratagene, San Diego, CA) from day 17 chick embryo (E17) brain RNA, as previously described.[25] Three screening protocols were used: (1) Clones were screened with a cocktail of probes of the rat nicotinic receptor gene family--the insert of clone pR11,

which contains fragments of the α2 gene;[30] the α3 gene[27] (λ PCA48, a gift of Dr. Jim Boulter); and the subcloned insert of the λ clone (a gift of Dr. Steve Heinemann), containing the α4-1 gene.[29] Hybridization was performed at 58°C in 5XSSPE (1XSSPE is 180 mM NaCl, 1 mM EDTA, 10 mM $NaPO_4$, pH 7.4), followed by washing at 60°C in 5XSSPE and at room temperature in 0.3XSSPE. (2) clones were screened with pCh20.2, a full-length chicken receptor structural subunit probe.[25] Hybridization was in 50% formamide, 5XSSPE, 42°C, and washing in 1XSSPE, 65°C. (3) Clones were screened with rat α3. Hybridization was in 30% formamide, 5XSSPE, 42°C, and washing in 1XSSPE, 65°C.

Isolated cDNA clones were analyzed by restriction mapping and DNA sequencing by a modified dideoxy chain termination method[85] using Sequenase enzyme (United States Biochemicals).

A system utilizing the λ P_R and P_L promoters was utilized to express in E. coli the putative cytoplasmic loop of the chicken brain receptor 75,000 molecular weight acetylcholine-binding subunit.[86] A Bsph1-PvuII fragment of the pCh26.1 clone encoding amino acids met335-ala517 was subcloned into the expression vector pJLA602, which had been digested with BamH1, blunt-ended with Klenow polymerase, and further digested with Nco1. The vector construct was verified by DNA sequencing. Expression of the protein was performed using the E. coli strain DH5α. Cultures were grown in 2XYT medium[72] at 28°C until an A_{600nm} of 0.4-0.5 was reached. The cultures were then shaken at 42°C for 2 hours to induce expression. Cultures were pelleted in a microfuge, resuspended in SDS-polyacrylamide gel electrophoresis sample buffer, boiled, and aliquots sampled for analysis by 15% SDS-polyacrylamide gel electrophoresis and Western blotting, using previously described techniques.[20] For large-scale preparation of expressed protein, 150 ml of induced culture was pelleted by centrifugation at 5000g for 15 minutes. The pellet was washed in 50 ml of 50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, and then resuspended by Polytron homogenization in 5 volumes of 10 mM NaCl, 100 mM KF, 5 mM EDTA, 5 mM EGTA, 5 mM iodoacetamide, 5 mM benzamidine, 1 mM PMSF, 2% SDS, then shaken for 1 hour at room temperature. The extract was centrifuged at 140,000g for 1 hour and the supernatant collected, diluted 5 fold in 100 mM NaCl, 10 mM $NaPO_4$, pH 7.5, 0.5% Triton X-100 (PBS Triton) containing 5 mM EDTA and 5 mM EGTA, and then recirculated for 14 hours at 4°C through 3 ml of mAb 289 coupled to AFC gel (New Brunswick Scientific), at a concentration of 8 mg mAb/ml of gel. The affinity column was washed with 50 ml of PBS Triton, 50 ml of 1 M NaCl, 10 mM $NaPO_4$, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, and the bound protein eluted with 4 column volumes of 50 mM Na Citrate, pH 3.0, 0.1% Triton X-100. The eluate was neutralized and then lyophilized.

A bacterial expression system based on one described by Rosenberg and Studier[63,64] was used to express fragments of chicken α3 subunits, putative α-bungarotoxin-binding protein subunits, and other receptor subunits. A fragment corresponding

to the large putative cytoplasmic loop of α3 leu323-met571 (numbered as in Figure 13) was prepared by partially digesting the α3 clone with HphI and completely digesting with NcoI. The ends were blunt ended with T4 polymerase in the presence of dNTP, and then the fragment was isolated using electrophoresis on agarose. The fragment was subcloned into pBluescript KS (Stratagene) previously linearized with SmaI, yielding the clone pCh35.2, having the BamHI site of the pBluescript KS polylinker next to the 5' end of the α3 gene fragment. A BamHI Eco R1 digest of pCh35.2 yielded a suitable fragment for an expression vector. It was cloned into a pET3c-derived vector, yielding clone Ch35.4. Cloning sites were confirmed by DNA sequencing. The expressed protein should have 15 N-terminal amino acids encoded by the vector, 127 amino acids of α3, and another 7 vector amino acids for a deduced molecular weight of 17,000 and a pI of 4.5. For expression, pCh35.4 was transformed into BL21(D3). From a single plaque an overnight culture was started in 3 ml. The next day this was expanded to 150 ml. At OD_{600}=0.8 expression was induced with 3 mM isopropyl-β-D-thioglactopyranoside. After 2 hours the culture was harvested by centrifugation and the pellet was resuspended in 5 ml of 50 mM Tris, pH 8.0, 10 mM EDTA, 0.5 mM PMSF, and frozen. An uninduced culture was similarly harvested as a control. Electrophoresis on acrylamide gels in SDS revealed a predominant band corresponding to an approximately 17,000 molecular weight peptide, only in the induced culture.

Expressed protein was purified from inclusion bodies as follows. To the thawed culture was added 15 ml of 50 mM Tris, pH 8.0, 10 mM EDTA, 1% Triton X-100, 0.5 mM PMSF. Sonication for three bursts of 15 seconds completed lysis. After centrifugation for 30 minutes at 20,000 rpm in a Beckman 50.2 Ti rotor at 4°C, the supernatant was discarded. The pellet was extracted with 3M NaSCN in 100 mM NaCl, 10 mM Na phosphate, pH 7.5, 0.2 mM PMSF, and centrifuged as before. This pellet was extracted with 0.5 M urea in the same buffer and centrifuged again. This pellet was solubilized in 8M urea, 100 mM NaCl, 10 mM Na phosphate, pH 7.5, 1 mM dithiothreitol. Dialysis against 100 mM NaCl, 10 mM Na phosphate, pH 7.5, resulted in partial precipitation. The resulting material appeared about 90% pure by electrophoresis.

Autoradiographic Localization of Receptors in Finch Brains

Intact adult male and female zebra finches (Poephila guttata) aged 6-12 months were killed by metofane overdose and decapitated. Their brains were immediately removed, submerged in ice-cold Tissue-Tek Frozen Embedding Medium (Miles Laboratories, Naperville, IL), and frozen in a -20°C freezer. The blocks were cryostat sectioned at 14 μm and sections were thaw mounted onto subbed slides and stored on ice until a complete brain was sectioned. The sections were freeze-dried in desiccator boxes in an ice-salt bath. Drops of solution containing one of the nicotinic ligands were applied to each set of dried sections at room temperature. Twenty nM ^{125}I-α-bungarotoxin in bovine serum was placed on sections for 30 minutes. The ^{125}I-mAbs were used

at a concentration of 2.0 nM with specific activities of 2-5 x 10^{18} cpm/mol. Labeling was done in 10^{-2} M HEPES buffered with Eagle's solution for 2 hours. Specificity of binding was determined in the case of α-bungarotoxin by including 1 mM unlabeled toxin, 1 mM carbamylcholine or 10 mM d-tubocurarine in the incubation mix; all three competitors reduced the binding of α-bungarotoxin to background levels and no regional variation was seen. Inclusion of excess unlabeled mAb 35 blocked the binding of ^{125}I-mAb 35, and unlabeled mAb 270 blocked the binding of ^{125}I-mAb 270. Following incubation, brain sections were washed for 1 hour, fixed in formaldehyde vapor, and applied to LKB (Pharmacia LKB, Piscataway, NJ) Ultrofilm for autoradiography. Following LKB autoradiography, slides incubated with each ligand were selected from each of four male brains and coated with Kodak (Rochester, NY) NTB-2 emulsion, overexposed, developed, and stained with cresyl violet to confirm the neuroanatomical localization of labeled regions.

A brain structure was judged to be labeled if it was visibly more labeled than ectostriatum (E)-a lightly labeled region using all three ligands. For small medullary structures, microscopic examination was necessary to evaluate silver grain density. A structure was judged to be labeled if it contained discernibly higher grain density than white matter in adjacent regions (such as fasciculus longitudinalis medialis and leminiscus medialis), or if silver grains were found clustered around nissl-stained cell nuclei. A qualitative estimate of the relative intensity of labeling in various brain structures was made separately for each ligand. No quantitative comparison of labeling intensity between ligands was made for any brain structure, so comparisons of labeling intensity between ligands are valid only when a structure is heavily labeled by one ligand and lightly by another ligand.

Histochemical Localization of Receptors in Chicken Retinas

A total of 37 white leghorn chicks from 1-14 days old were used in this study. The chicks were killed with an overdose of ketamine and xylazine. The eyes were rapidly removed, the anterior pole and vitreous cut away, and the eye was immersed in ice-cold 0.1%, 1%, or 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.4. After 15 minutes-24 hours, the tissue was placed in a solution of 30% sucrose in 0.1 M phosphate buffer for at least 12 hours. The eyes were then frozen in embedding medium. Sections, 10 μm thick, were cut on a cryostat and collected on gelatin-coated glass slides. Alternatively, radial incisions were made in the retina so that it could be flattened. It was then frozen, and 30-μm-thick sections were cut parallel to the vitreal surface on a sliding microtome. Sections cut in this plane will be referred to as "horizontal sections." These sections were processed "free floating" in small vials. In one case, 20-μm-thick sections were cut perpendicular to the vitreal surface on a cryostat and immersed in buffer solution. They were processed in the same fashion as horizontal sections.

The indirect fluorescence and avidin-biotin techniques were used in this study. In some instances a "double level" fluorescence technique was used, which allowed the visualization of two different immunohistochemical markers in the same section through the use of two different fluorophores.

The sections were washed in three changes of phosphate buffer and then incubated with the primary antibodies diluted in phosphate buffer. The mAbs were used at a concentration of 10-20 nM and the antiserum to choline acetyltransferase (ChAT) was used at a dilution of 1:1000. The incubation buffer included 0.3% Triton X-100 and 0.05% NaN_3, and sections were incubated for 12-24 hours at a temperature of 4°C. The tissue was next washed in three changes of buffer and then incubated in either fluorescein- or rhodamine-isothiocyanate (FITC or RITC, respectively) conjugated goat anti-rat IgG (Boehringer Mannheim) diluted 1:100 in buffer. The sections were subsequently washed in several changes of buffer and coverslipped with a mixture of carbonate buffer and glycerine.

As a determination of secondary antiserum specificity, we processed some sections for immunofluorescence, but omitted the primary antibody. Other sections from the same retina were processed normally in parallel. Those sections that had not been exposed to primary antibody exhibited no staining.

Tissue to be double-labeled with antiserum directed against ChAT and nicotinic receptor antibodies was processed according to the procedure used by Erichsen et al.[87] The antibody directed against ChAT was generously supplied by Miles Epstein and Carl Johnson and has been previously characterized.[88] For these double-label studies the antiserum and the antibody were mixed and applied to the tissue for times similar to those stated above. The tissue was then washed in three changes of phosphate buffer, and a mixture of goat anti-rabbit IgG conjugated to FITC and goat anti-rat IgG conjugated to RITC was applied for 1 hour. In some cases the fluorophores were reversed.

Sections to be processed with the avidin-biotin technique were incubated in primary antibody, washed in three changes of phosphate buffer, and placed in a solution of biotinylated goat anti-rat IgG (Vector Laboratories, Burlingame, CA) diluted 1:200 in 0.3% Triton X-100 in buffer for 1 hour. The sections were then washed and incubated with a mixture of biotin and avidin-bound-horseradish peroxidase diluted 1:100 in phosphate buffer with 0.3% Triton for 1 hour. Following washes in buffer, the tissue was incubated with 0.05% diaminobenzidine in phosphate buffer for 15 minutes. Hydrogen peroxide was then added to make a final concentration of 0.01% and the solution was gently snaken. The reaction was allowed to proceed for 15 minutes. The tissue was then washed in several changes of buffer, cleared, and coverslipped.

The sections were examined with a microscope fitted for epifluorescence microscopy or differential interference microscopy.

Histological Studies of Nicotinic Receptors in Frog Brains

Most of the experiments were done on Rana pipiens (body length of 5.0-7.5 cm, either sex) obtained from Hazen Company (Alburg, VT).

Biotinylated rabbit anti-rat IgG and avidin-biotinylated horseradish peroxidase complex were purchased in kit form (Vectastain) from Vector Laboratories. Fluorescein-goat-anti-rat IgG was obtained from Cappel Laboratories (Organon Teknika, Malvern, PA). All other reagents were purchased from Sigma Chemical Company unless indicated otherwise.

All surgical procedures were performed following anesthesia by immersion of animals in 2 mM tricaine methanesulfonate. The optic nerve was cut after approaching it through the soft palate. The soft palate was then sutured with 6-0 monofiliment nylon thread. Retinas were eviscerated by aspiration following removal of the lens and vitreous body. The eyelid was then sutured shut. The projection of retinal ganglion cells to the tectum was labeled by placing crystals of horseradish peroxidase (Sigma, Type VI) in contact with the central stump of the severed optic nerve and examining the tectum 2-3 days later.

Light microscopic immunocytochemistry was performed on frozen or vibratome sections of tecta of animals fixed by perfusion with periodate-lysine-paraformaldehyde (PLP) containing 2% paraformaldehyde or with 4% acrolein in 90 mM Na phosphate buffer. The avidin biotinylated-peroxidase complex (ABC) method of Hsu et al.[89] was used to visualize primary antibody binding, and peroxidase was revealed colorimetrically by the cobalt-glucose oxidase procedure of Itoh et al.[90]

Animals were perfused through the truncus arteriosus, first, with frog Ringer's containing 2 mM tricaine methanesulfonate until fluid returning to the heart was clear, and subsequently with fixative for 7.5 (acrolein) or 90 (PLP) minutes. The brain with optic nerves was then removed and immersion-fixed for an additional 7.5 (acrolein) or 30 (PLP) minutes. For frozen sections the brain was then equilibrated in 30% sucrose in 90 mM Na phosphate buffer, frozen by immersion in liquid nitrogen, and mounted in Tissue Tek mounting medium (Miles Laboratories) prior to sectioning at 10 μm in a microtome cryostat. Sections were placed on subbed glass slides and air-dried for 30 minutes before use. For vibratome sections, the brain was embedded in 4% agar and sectioned at 100 μm. In a separate set of experiments, retinas were vibratome-sectioned after being removed from the sclera of an anesthetized frog and immersion fixed in PLP for 30 minutes.

Vibratome sections of tissue were processed "free-floating" according to following schedule (all steps were done at room temperature unless indicated otherwise). Frozen sections were processed mounted and for shorter times (1 hour for antibody incubations, 30 minutes for washes).

1. Preincubate vibratome sections in frog Ringer's containing 3% normal rabbit serum and 0.025% saponin (RRS) for 30 minutes.

2. Incubate sections in primary antibody diluted to a titer of 10-200 nM in RRS for 16 hours at 4°C.

3. Wash in RRS for 2-3 hours, changing the solution every 20 minutes.

4. Incubate the sections in biotinylated rabbit anti-rat IgG diluted 1:200 in RRS for 6 hours.

5. Wash in RRS for 2-3 hours, changing the solution every 20 minutes.

6. Incubate sections in avidin-HRP, diluted 1:100 in RRS, for 16 hours at 4°C.

7. Wash in RRS for 1 hour, changing the solution every 20 minutes.

8. Wash in Ringer's for 1 hour, changing the solution every 20 minutes.

9. Fix the sections in 1% glutaraldehyde in 60 mM sodium phosphate buffer, pH 7.2, for 1 hour.

10. Rinse in Ringer's, 15 minutes.

11. Preincubate the sections in a solution containing diaminobenzidine, nickel ammonium sulfate, cobalt chloride, and β-D-glucose in phosphate buffer (final concentrations: diaminobenzidine, 0.5 mg/ml; nickel ammonium sulfate, 0.025%; cobalt chloride, 0.025%; β-D-glucose, 2 mg/ml) for 15 minutes.

12. Incubate the sections in a solution identical to the preincubation solutions, but containing in addition 0.2 mg/ml ammonium chloride and 0.003 mg/ml glucose oxidase (Sigma, Type II) until there is adequate staining intensity.

13. Rinse the sections in Ringer's for 15 minutes.

14. Mount the sections in 90% glycerol/10% Ringer's.

In a few instances immunoperoxidase experiments were performed on only one side of the tectum to compare the pattern of staining with the pattern of the retinotectal projection (the

retinotectal projection was almost completely crossed). Horseradish peroxidase crystals were placed in contact with the central cut end of the left optic nerve in an anesthetized frog. Two to three days later the animal was reanesthetized and perfused with fixative. The tectum was then serially sectioned, with the plane of the sections being as close as possible to coronal. Each section was then split in the mid-saggital plane and each right half-section was set aside. The left half-sections were reacted for receptor (through step 10, above), and both right and left halves were reacted for horseradish peroxidase. The matching sections were then reassembled photographically, thus producing a reconstituted tectal slice in which the retinal projection appeared on the right half and the receptor immunoreactivity appeared on the left half.

The procedure for performing immunocytochemical staining for analysis in the electron microscope was somewhat similar to that for light microscopy. Only vibratome sections were used, and saponin was omitted to preserve adequate fine structure. Electron microscopic analysis was performed after cutting thin sections orthogonal to the plane of the vibratome section. The heaviest peroxidase stain was found at the surface of the vibratome section, but the tissue was poorly preserved. The best results were obtained 1-3 μm into the section, where peroxidase staining was still strong and where tissue preservation was adequate.

Electron microscopic analysis was performed on sections treated as above (steps 1-13) and postfixed in 1% OsO_4 in 90 mM sodium phosphate buffer, pH 7.2, dehydrated and embedded in Epon/Araldite. Sections having a silver interference color (70-80 nm) were cut on an ultramicrotome and examined without grid staining on an electron microscope. Contrast was sometimes enhanced by adding tannic acid to the glutaraldehyde (step 9, above) to a final concentration of 0.3%.

In a few experiments vibratome sections of tectum were incubated in concentrated solutions of the tracer ferritin or 6 nm colloidal gold-protein A. Cationized ferritin was used at 20 mg/ml in Ringer's. Colloidal gold-protein A was prepared according to the technique of Muehlpfordt,[91] conjugated to protein A and purified after Slot and Geuze,[92] and used at an OD @ 525 nm of 1.0 in Ringer's. Tissue incubated for 1 hour with tracer was fixed in 1% glutaraldehyde in 60 mM Na phosphate without tracer washout. The purpose of these experiments was to learn the extent to which these reagents penetrated the vibratome slice and the cell profiles within its interior.

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